ORGANIZATION OF BRAIN AND SPINAL CORD LOCOMOTOR NETWORKS IN LARVAL LAMPREY

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ABSTRACT

In vertebrates, rhythmic locomotor behaviors, such as walking, flying, and swimming, are initiated by "command" systems in the brain that activate locomotor networks in the spinal cord to initiate rhythmic motor activity and locomotor movements (Grillner, 1981). The spinal locomotor networks are distributed along the spinal cord and coupled by a spinal coordinating system to form a central pattern generator (CPG). Spinal CPGs are capable of producing the basic pattern of rhythmic activity in the absence of sensory feedback, although sensory feedback is critical for "fine tuning" the centrally generated locomotor pattern (Grillner, 1975).

In *in vitro* brain/spinal cord preparations from larval lamprey, spinal locomotor activity that is similar to swimming activity in whole animals can be initiated by pharmacological microstimulation in several brain areas: rostrolateral rhombencephalon (RLR); dorsolateral mesencephalon (DLM); ventromedial diencephalon (VMD); and reticular nuclei. However, the actual movements that would result from this *in vitro* burst activity have not been investigated in detail. In the present study, pharmacological stimulation was applied to the above brain locomotor areas in semi-intact preparations from larval lamprey, and swimming movements and muscle burst activity in the freely moving caudal body were analyzed (Jackson and McClellan, 2001; Jackson et al., 2006). First, bilateral stimulation with pharmacological agents in the VMD, DLM, or RLR initiated symmetrical swimming and well-coordinated muscle burst activity that was not significantly different than those during swimming in whole animals. Unilateral stimulation in these areas usually did not produce symmetrical swimming. Second, with synaptic transmission blocked in the brain, bilateral stimulation in reticular nuclei also

initiated symmetrical swimming movements and muscle burst activity. In contrast, unilateral stimulation in reticular nuclei elicited either asymmetrical undulatory movements or flexure movements.

The above brain areas initiate well-coordinated spinal locomotor activity, but the sizes and pharmacology of these areas are unknown. Also, certain aspects of the organization of brain locomotor command areas in the lamprey have not been fully investigated. In the present study, pharmacological microstimulation with excitatory amino acids (EAAs) or their agonists were used to map the sizes, pharmacology, and organization of the above brain locomotor areas (Jackson and McClellan, 2001; Jackson et al., 2006). First, mapping experiments indicate that the RLR, DLM and VMD locomotor areas are located in relatively discrete areas of the brain. In addition, stimulation as little as 50 μm outside of these areas was ineffective and elicited tonic or uncoordinated motor activity. Second, pharmacological stimulation with NMDA, kainate, or AMPA in the VMD or DLM reliably initiated well-coordinated spinal locomotor activity. In the RLR, stimulation with all three EAA agonists could initiate spinal locomotor activity, but NMDA was more reliable than kainate or AMPA. Thus, all three higher order brain locomotor areas appear to contain neurons with receptors for EAAs. Third, with synaptic transmission blocked only in the brain, stimulation in the RLR, VMD, or DLM no longer initiated spinal locomotor activity, suggesting that these higher order locomotor areas do not directly activate spinal locomotor networks. Fourth, following a complete transection at the mesencephalon-rhombencephalon border, stimulation in the RLR no longer initiated spinal locomotor activity, suggesting that more rostral neural structures (e.g. VMD, DLM) are necessary for RLR-initiated locomotion.

In the third part of this study, the roles of reciprocal connections between left and right spinal CPG modules in larval lamprey were examined in whole animals and *in vitro* brain/spinal cord preparations (Jackson et al., 2005). Instead of activating spinal locomotor networks by bath-applied pharmacological agents or by non-specific electrical stimulation of the surface of the spinal cord, motor activity was initiated in a more physiological fashion from the brain and recorded in both intact and lesioned regions of spinal cord. With longitudinal midline lesions in the rostral or caudal spinal cord, isolated left and right hemi-spinal cords, in the absence of connections with intact regions of cord, are not able to generate locomotor burst activity in response to descending activation from locomotor command systems in the brain. Thus, in larval lamprey commissural interneurons that couple right and left spinal locomotor networks appear to contribute to left-right phasing of burst activity as well as rhythmogenesis.

In summary, results from the present study strongly suggest that ventral root activity initiated from brain locomotor areas in *in vitro* preparations underlies locomotion. In addition, the results indicate that bilateral stimulation is a more physiologically realistic test of the function of these brain areas than unilateral stimulation, which is used in most studies. These studies also provide additional support for a model of parts of the brain command system for locomotion in the lamprey (Paggett et al., 2004). Furthermore, the general organization of brain locomotor areas in the lamprey appears to be similar to those found in "higher" vertebrates. Finally, in larval lamprey, reciprocal connections between left and right spinal locomotor networks are critical not only for phasing of burst activity but also rhythmogenesis of the locomotor pattern itself.

CHAPTER 1

INTRODUCTION

GENERAL INTRODUCTION

In nature, the ability to locomote around in the environment in search of food or the avoidance of predators is crucial to the survival of the animal. Subsequently, one of the most fundamental questions in neurobiology is how rhythmic locomotor behaviors, such as swimming, flying, and walking, are initiated and coordinated. In a wide variety of vertebrates, locomotor systems appear to have a similar general organization (reviewed in Stein, 1978; Grillner, 1981; McClellan, 1986,1996). Higher order "command" or " initiation" centers located in the brain activate locomotor networks in the spinal cord that are coupled by a spinal coordinating system to form a central pattern generator (CPG) (Fig. 1). Once activated, the spinal CPG is capable of producing the basic locomotor pattern in the absence of sensory input. However, sensory feedback plays an important role in modulating the CPG and "fine tuning" rhythmic locomotor activity (Orlovsky, 1972; Grillner, 1981; McClellan, 1986).

The lamprey, a primitive vertebrate, has a number of advantages that make it amenable for studying the initiation and coordination of locomotion. First, the lamprey has a comparatively simple central nervous system (CNS) that retains the same basic CNS features found in the nervous systems of more complex vertebrates (Niewenhuys, 1977). Second, the brain is relatively thin (~400 μM in larval animals) and is not a significant diffusion barrier to gas exchange and nutrients, and *in vitro* brain/spinal cord preparations can be viable for 2-3 days when kept in chilled (4-7°C) Ringer's solution.

Third, the lamprey brain contains several large identifiable Müller and Mauthner cells that are easily accessible from the ventricular surface and are useful for certain types of studies. Finally, in the lamprey, locomotor (i.e. swimming) behavior is characterized by body undulations that increase in amplitude with increasing distance from the head (Davis et al., 1993; also see Grillner and Kashin, 1976). Locomotor activity is characterized by left-right alternation of burst activity at the same segmental level and a rostrocaudal phase lag for ipsilateral burst activity (Wallen and Williams, 1984).

In the present studies, *in vitro* brain/spinal cord preparations, semi-intact preparations, and whole larval lamprey were used to investigate the following questions: (1) Does the pattern of spinal locomotor activity initiated by bilateral pharmacological microstimulation in higher order locomotor areas in the lamprey brain underlie swimming in whole animals? (2) What is the size and pharmacology of the higher locomotor areas that initiate spinal locomotor activity? (3) Is reciprocal coupling between right and left spinal locomotor networks necessary for rhythmogenesis or phasing of spinal locomotor activity?

LOCOMOTOR MOVEMENTS INITIATED BY PHARMACOLOGICAL STIMULAITON IN HIGHER LOCOMOTOR COMMAND AREAS IN LARVAL LAMPREY (CH. III)

1. *Background*

Locomotor command systems appear to have a similar organization in a wide range of vertebrates (reviewed in McClellan, 1986). For example, locomotor activity can be initiated by pharmacological or electrical microstimulation in a number of "brain

locomotor areas" that are present in different vertebrates. First, in a variety of vertebrates electrical or pharmacological stimulation in reticular nuclei activates reticulospinal (RS) neurons that are thought to directly activate spinal CPGs and initiate spinal locomotor activity (Steeves et al., 1987; Noga et al., 1988; Livingston and Leonard, 1990; Atsuta et al., 1990; Kinjo et al., 1990; Garcia-Rill et al., 1990; Bernau et al., 1991; Perreault et al., 1993; Sholomenko and O'Donovan, 1995; also see Jackson and McClellan, 2001). Second, focal electrical stimulation in the pontomedullary locomotor strip (PLS), which is in the vicinity of the spinal nucleus of the trigeminal nerve and probably sensory in nature, elicits well-coordinated spinal locomotor activity (Mori et al., 1977; Kazennikov et al., 1981; Garcia-Rill et al., 1983; Selionov and Shik, 1984; Steeves et al., 1987; Bayev et al., 1988; Beresovskii and Bayev, 1988; Noga et al., 1988, 1991; Sholomenko et al., 1991). Third, electrical or pharmacological stimulation in a specific area in the mesencephalon, called the mesencephalic locomotor region (MLR), evokes both spinal locomotor activity and locomotor movements in a wide variety of vertebrates (Shik et al., 1966; Kashin et al., 1974, 1981; Eidelberg et al., 1981; Parker and Sinnamon, 1983; Sinnamon, 1984; Grillner and Wallen, 1984; McClellan and Grillner, 1984; Amemiya and Yamaguchi, 1984; Garcia-Rill and Skinner, 1987; Milner and Mogenson, 1988; Coles et al., 1989; McClellan, 1990; Bernau et al., 1991; Sholomenko et al., 1991; Douglas et al., 1993; Fetcho and Svoboda, 1993; Uematsu and Todo, 1997; Sirota et al., 2003; Cabelguen et al., 2003). Fourth, stimulation in the subthalamic locomotor region (SLR) evokes well-coordinated locomotor activity (Orlovsky, 1969; Parker and Sinnamon, 1983; Skinner and Garcia-Rill, 1984). In rats, locomotor activity can be elicited by electrical stimulation in the lateral hypothalamus (Sinnamon, 1984; Sinnamon

and Stopford, 1987; Sinnamon, 1990). Finally, in the cat, locomotor activity also can be initiated by focal microstimulation in the cerebellar locomotor region (CLR), which corresponds to the fastigial nucleus (Mori et al., 1999).

In several vertebrates, the functional connectivity between certain brain locomotor areas has been examined in some detail (Orlovsky, 1970; Garcia-Rill and Skinner, 1987a,b; Levy and Sinnamon, 1990; Bernau et al., 1991; Noga et al., 1991). For example, lesions or blocking neuronal activity in medullary reticular nuclei can abolish MLR- or PLS-initiated locomotor activity (Shefchyk et al., 1984; Garcia-Rill and Skinner, 1987a; Skinner et al., 1990; Bernau et al., 1991; Noga et al., 1991,2003). In addition, stimulation in the MLR or SLR elicits synaptic potentials in RS neurons (Orlovsky, 1970; Garcia-Rill and Skinner, 1987b). Finally, locomotor activity initiated from the hypothalamus can be blocked by injection of anesthetics in midbrain sites (Levy and Sinnamon, 1990).

In the lamprey, spinal locomotor activity in *in vitro* preparations and swimming movements in semi-intact preparations can be initiated by pharmacological or electrical microstimulation in several specific areas of the brain (Fig. 2B,4): reticular nuclei, including the anterior (ARRN), middle (MRRN), and posterior (PRRN) rhombencephalic reticular nuclei (Hagevik et al., 1996; Jackson et al., 2001); ventral thalamus or sometimes called the "diencephalic locomotor region" (DLR) in the ventromedial diencephalon (VMD; El Manira et al., 1997; Paggett et al., 2004); dorsolateral mesencephalon (DLM; Paggett et al., 2004); mesencephalic locomotor region (MLR; McClellan and Grillner, 1984; Sirota et al., 2000); and rostrolateral rhombencephalon (RLR; Hagevik and McClellan, 1994; McClellan, 1994; Hagevik et al., 1996, Paggett et

al., 2004). Unilateral electrical microstimulation in the MLR elicits "controlled" swimming in which the frequency of burst activity increases with increasing stimulus intensity (McClellan and Grillner, 1984; McClellan, 1989; Sirota et al., 2000). Also, a single brief, unilateral ejection of AMPA in the MLR initiates bouts of swimming lasting up to two minutes (Sirota et al., 2000). In contrast, in semi-intact preparations, unilateral electrical stimulation in the MRRN and ARRN was reported to occasionally elicit only a few cycles of swimming that quickly deteriorated into tonic contractions, while similar stimulation in the PRRN was said to elicit spastic muscle contractions (Sirota et al., 2000).

2. *Purpose*

In *in vitro* brain/spinal cord preparations from larval lamprey, pharmacological stimulation in higher order locomotor areas (RLR, VMD, or DLM) initiates locomotor-like ventral burst activity whose parameters are similar to those of muscle burst activity during swimming in whole animals (Davis et al., 1993; McClellan, 1994; Boyd and McClellan, 2002; Paggett et al., 2004). However, intersegmental phase lags of *in vitro* burst activity are shorter than those during swimming in whole animals, suggesting that mechanosensory feedback may contribute to shaping motor activity (Hagevik and McClellan, 1994; McClellan, 1994). During normal locomotion, the brain locomotor areas probably are active bilaterally rather than unilaterally, and thus, bilateral stimulation in these areas is a more physiological method of initiating locomotion. A detailed analysis of the movements that would be initiated by bilateral pharmacological stimulation in higher order locomotor areas (RLR, VMD, or DLM) or reticular nuclei has not been performed in lamprey. In particular, it is not known if brain-initiated *in vitro*

burst activity would result in normal swimming behavior, including symmetrical swimming movements and caudally propagated undulations increase in amplitude with increasing distance from the head.

3. *Brief Summary of Results*

In the present study in semi-intact preparations from larval lamprey, bilateral pharmacological microstimulation was applied to specific brain locomotor areas (see Methods) to initiate muscle activity and swimming movements, which were compared to those elicited by unilateral pharmacological stimulation and during swimming in whole animals. Bilateral pharmacological stimulation in higher order locomotor areas (RLR, DLM, or VMD) or reticular nuclei initiated symmetrical swimming movements and muscle burst activity that were not significantly different than those during swimming in whole animals. In contrast, unilateral stimulation in these brain locomotor areas usually elicited asymmetrical movements. Results from the present study strongly suggest that brain-initiated locomotor activity in *in vitro* preparations underlies locomotor behavior. In addition, since bilateral pharmacological stimulation could initiate symmetrical, well-coordinated locomotor movements while unilateral stimulation usually did not, bilateral stimulation probably is a more physiological test of the function of brain locomotor areas. Finally, this study is one of the few to directly compare command system-initiated locomotion and swimming activity in whole animals and semi-intact preparations with *in vitro* spinal locomotor activity ("fictive" locomotion). Preliminary accounts of these data have appeared in abstract form (Jackson and McClellan, 2001; Jackson et al., 2006).

SIZE, PHARMACOLOGY, AND ORGANIZATION OF HIGHER LOCOMOTOR COMMAND AREAS IN LARVAL LAMPREY (CH. IV)

1. *Background*

Excitatory amino acids (EAAs) have been implicated as neurotransmitters in the brain locomotor areas of several vertebrates (McClellan, 1986,1994; Brudzynski et al., 1986; Milner and Mogenson, 1988; Noga et al., 1988; Livingston and Leonard, 1990; Kinjo et al., 1990; Garcia-Rill et al., 1990; Sholomenko et al., 1991a; Hagevik and McClellan, 1994a,b). EAAs are thought to act on at least three different types of ionotropic receptors that are defined by their agonists (Mayer and Westbrook, 1987; Watkins and Olverman, 1987; Krogsgaard et al., 1992): NMDA (N-methyl-D-aspartate); KA (kainate); and AMPA (α-amino-5-hydroxy-3-methyl-4-isoxazole propionic acid).

In the lamprey, spinal locomotor activity can be initiated by pharmacological or electrical microstimulation in several brain locomotor areas (RLR, VMD, DLM, reticular nuclei) that appear to be similar to those found in "higher" vertebrates (see above). In the lamprey, during RLR-initiated locomotor activity, blockade of neuronal activity in the DLM or VMD abolishes or greatly attenuates the spinal locomotor rhythm, suggesting that neurons in the RLR project rostrally to the DLM and VMD locomotor areas (Paggett et al., 2004). Additional results suggest that neurons in higher locomotor areas in the VMD and DLM project directly to RS neurons (Paggett et al., 2004). For example, VMD- or DLM-initiated locomotor activity is abolished or substantially attenuated when neural activity is focally blocked in reticular nuclei. Furthermore, electrical stimulation in the VMD or DLM can elicit monosynaptic responses in RS neurons (Paggett et al., 2004; El Manira et al, 1997). Results from other studies suggest that neurons in the MLR

also project directly to reticular nuclei (Sirota et al., 2000; also see McClellan, 1989). These results lead to the following working model for part of the locomotor command system in the lamprey brain (see Fig. 33) (Paggett et al., 2004):

RLR → DLM and VMD → RS neurons → spinal CPGs

Preliminary results suggest that second order trigeminal sensory neurons may project to the RLR, but this remains to be proven. In addition, this model does not include all aspects of the command system for swimming. For example, other sensory modalities (e.g. vision, olfaction) are not included but appear to have inputs to the command system, and other brain locomotor areas (e.g. MLR) have been omitted because the inputs to these regions have not been determined.

2. *Purpose*

Several aspects of the organization of the brain locomotor command system remain to be investigated. First, although preliminary results suggest that the DLM, RLR, and VMD locomotor areas are restricted (Hagevik et al., 1996), a systematic mapping of these areas has not been performed. Second, the specific subtypes of ionotropic EAA receptors within the above locomotor areas and their contributions to initiation of locomotion are not known. Third, blocking neural activity in reticular nuclei often, but not always, abolishes or attenuates VMD- or DLM-initiated locomotor activity (Paggett et al., 2004). Thus, in theory, there is the possibility of parallel pathways from the VMD or DLM directly to the spinal cord. Finally, other studies suggest that in the lamprey, trigeminal sensory inputs evoke locomotion by a disynaptic pathway to RS neurons (Viana Di Prisco et al., 1997). Although RLR-initiated spinal locomotor activity is usually abolished or attenuated when neuronal activity is focally blocked in the DLM

or VMD, we have not excluded the possibility that the RLR also can directly activate RS neurons or directly activate spinal CPGs to initiate locomotor activity.

In the present study, the above aspects of the brain locomotor command system were investigated using *in vitro* brain/spinal cord preparations from larval lamprey. Experiments were performed to determine the sizes and pharmacology (i.e. EAA receptors) of the DLM, RLR, and VMD locomotor areas. In addition, synaptic transmission was blocked in the brain to determine if these locomotor areas could directly activate spinal locomotor networks. Finally, lesion experiments were performed to determine if RLR-initiated spinal locomotor activity requires more rostral brain structures or can be evoked by pathways entirely within the rhombencephalon.

3. *Brief Summary of Results*

For this part of the study, pharmacological microstimulation with excitatory amino acids (EAAs) or their agonists was used to map the sizes and pharmacology of the above brain locomotor areas. First, mapping experiments indicate that the RLR, DLM and VMD locomotor areas are located in discrete areas of the brain. In addition, stimulation as little as 50 μm outside of these areas was ineffective and elicited tonic or uncoordinated motor activity. Second, pharmacological stimulation with NMDA, kainate, or AMPA in VMD or DLM reliably initiated well-coordinated spinal locomotor activity. In the RLR, stimulation with all three EAA agonists could initiate spinal locomotor activity, but NMDA was more reliable than kainate or AMPA. Thus, all three higher order brain locomotor areas appear to contain neurons with receptors for EAAs. Third, with synaptic transmission blocked only in the brain, stimulation in the RLR, VMD, or DLM no longer initiated spinal locomotor activity, indicating that these higher

order locomotor areas do not directly activate spinal locomotor networks. Fourth, following a complete transection at the mesencephalon-rhombencephalon border, stimulation in the RLR no longer initiated spinal locomotor activity. Thus, RLR-initiated locomotor activity requires more rostral neural structures, such as the VMD and DLM. These studies provide additional support for a model of parts of the brain command system for locomotion in the lamprey (Paggett et al., 2004). Preliminary accounts of this part of the thesis have appeared in abstract form (Hagevik et al., 1996; Paggett et al., 2001; Jackson et al., 2006).

DISRUPTION OF LEFT-RIGHT RECIPROCAL CONNECTIONS IN THE SPINAL CORD OF LARVAL LAMPREY (CH. V)

1. *Background*

For certain rhythmic behaviors such as locomotion, the CPGs consist of several "local control centers" or "modules" that are distributed in the spinal cords of vertebrates or ventral nerve cord ganglia of segmented invertebrates. The modules are coupled by a coordinating system, both between left and right sides of the CNS as well as longitudinally, to regulate the relative timing of motor patterns generated in different parts of the nervous system that control different regions of the body (reviewed in Skinner and Mulloney, 1998; Hill et al., 2003). It is thought that alternating locomotor activity, such as left-right alternation or flexor-extensor alternation, is generated by a "half-center" network consisting of two CPG modules that are connected by reciprocal inhibition. The degree to which CPGs can be divided into modules that are rhythmogenic and that can generate normal burst activity in isolation varies between animals.

In crustaceans, swimmerets are controlled by separate CPG modules, which are bilaterally distributed in several abdominal ganglia and which, when isolated from remaining neural circuitry, produce rhythmic swimmeret motor activity (Murchison et al., 1993). In *Clione*, a marine mollusk, dorsal-ventral swimming movements of the "wings" are controlled by CPG modules on right and left sides of the CNS, and each module alone can generate alternating dorsal-ventral swimming activity (reviewed in Arshavsky et al., 1998). In addition, many of the neurons that generate rhythmic dorsal or ventral motor activity function as endogenous oscillators when isolated. For leech swimming, single or short chains of ganglia from the ventral nerve cord can generate swimming-like motor activity (Hocker et al., 2000). However, for chains of ganglia, the frequency and intersegmental phase lags of the rhythm are highly dependent on the number of segments (Pearce and Friesen, 1984,1985) and sensory feedback (Cang and Friesen, 2002). The CPG circuits in isolated left or right hemi-ganglia are unable to generate swimming motor activity (Friesen and Hocker, 2001).

For locomotor behavior in quadrupedal vertebrates, distinct spinal locomotor generators produce the motor activity for forelimbs and hindlimbs, and each limb appears to be governed by a separate "local control center" (reviewed in Grillner, 1981). In the cat, isolated right or left lumbar hemi-spinal cord regions can produce locomotor movements of the corresponding hindlimb (Kato, 1990). In *in vitro* lumbar spinal cords from neonatal rats or mice following either sagittal midline spinal lesions, activation of only one side of the cord, or isolation of one side of the cord, right or left hemi-spinal networks generate rhythmic locomotor-like burst activity in response to bath-applied pharmacological agents (Kudo and Yamada, 1987; Tao and Droge, 1992; Bracci et al.,

1996; Cowley and Schmidt, 1997; Kjaefulff and Kiehn, 1997; Kremer and Lev-Tov, 1997; Bonnot and Morin, 1998; Whelan et al., 2000; Nakayama et al., 2002; also see Cheng et al., 1998). In the neonatal rat lumbar spinal cord, strychnine, a glycine receptor blocker, blocks left-right reciprocal inhibition and converts left-right alternating locomotor-like burst activity to synchronous bursting (Cowley and Schmidt, 1995; also see Jovanovic et al., 1999 for similar results in mudpuppy), suggesting that separate left and right spinal modules control each limb and that left-right reciprocal connections are largely involved in phasing of activity rather than rhythmogenesis. During development in embryonic rat spinal cord, synchronous left-right burst activity switches to alternating activity as the sign of left-right reciprocal connections changes from excitation to inhibition (Nakayama et al., 2002). Separate modules may also control flexor and extensor rhythmic burst activity, since strychnine converts flexor-extensor alternation to coactivation (Cowley and Schmidt, 1995). Finally, rhythmic flexor or extensor bursts can occur without antagonistic motor activity (Whelan et al., 2000; also see Cheng, 1998 for complementary results in mudpuppy), although it is not always clear whether the absence of motoneuron bursting signifies a lack of activity in interneurons in the corresponding module.

In the embryonic chick, the *in vitro* lumbosacral spinal cord generates spontaneous episodes of locomotor-like activity (O'Donovan, 1989). Following sagittal lesions in the lumbosacral spinal cord, left or right spinal motor circuitry is able to generate rhythmic burst activity (Ho and O'Donovan, 1993), suggesting that each limb is controlled by a separate module that can be rhythmogenic in the absence of reciprocal inhibition.

In the low spinal turtle, unilateral tactile stimulation of different areas of the lower body elicits various forms of the scratch reflex (e.g. rostral, pocket, or caudal scratch) in the ipsilateral hindlimb (reviewed in Stein et al., 1998), suggesting that the hindlimbs are controlled by separate left and right scratch rhythm generating modules. However, several results suggest that left or right scratch generating modules interact with and share circuitry with contralateral modules, a notion referred to as the "bilateral shared core" hypothesis (Stein et al., 1995,1998; reviewed in Stein et al., 1998). For example, following removal of the left half of the lower spinal cord (D7-S2 segments), stimulation of the right (left) receptive field for rostral scratching elicits rhythmic right hip flexor (extensor) bursts in the absence of antagonistic activity (Stein et al., 1995). Thus, in response to unilateral stimulation, contralateral spinal circuitry contributes to ipsilateral scratch motor pattern generation. Furthermore, rostral scratch motor patterns can occasionally occur in the absence of ipsilateral hip extensor activity, and stimulation of the contralateral midbody restores the missing parts of the pattern (Currie and Gonsalves, 1999). Since rhythmic flexor bursts can occur in the absence of extensor bursts, reciprocal inhibition between flexor and extensor modules does not appear to be required for rhythmogenesis of hip flexor modules (Stein et al., 1995,1998).

In most fish and some amphibians, swimming behavior and motor activity are produced by two components (Grillner and Kashin, 1976): (a) left-right bending of the body at each segmental level that is produced by left-right alternating muscle burst activity; and (b) caudally propagating body undulations that are produced by a rostrocaudal phase lag of ipsilateral muscle burst activity. The spinal CPG modules for swimming are distributed along the spinal cord and coupled by a coordinating system.

2. *Purpose*

In the lamprey, the mechanisms for rostrocaudal phase lags and left-right alternation of locomotor activity have been examined in some detail. First, as few as two or three spinal cord segments can generate swimming-like burst activity (reviewed in Buchanan, 2001). Both neurophysiological experiments and computer modeling suggest that rostrocaudal phase lags are largely determined by asymmetrical short-distance longitudinal coupling between spinal cord modules that is ipsilateral, excitatory, and stronger in the descending direction (Hagevik and McClellan, 1994; reviewed in McClellan, 1996). In contrast, long distance coupling between distant spinal CPG modules (McClellan and Hagevik, 1999) and a gradient of oscillator frequencies along the spinal cord (Hagevik and McClellan, 1999) do not appear to contribute significantly to the generation of rostrocaudal phase lags.

Second, left and right CPG modules in the lamprey spinal cord appear to be connected by reciprocal inhibition that appears to be mediated, in part, by crossed-contralaterally projecting interneurons (CCI's), a class of commissural interneurons (reviewed in Buchanan, 2001). In theory, reciprocal inhibition might contribute to motor pattern generation in at least two ways: (a) regulation of left-right phasing between rhythmogenic left and right unit oscillator modules; or (b) significant contribution to rhythmogenesis. Experiments to test these possibilities have lead to conflicting interpretations. In one study in which longitudinal midline lesions were made in *in vitro* spinal cord preparations from adult lamprey, motor circuitry in hemi-spinal cords generated rhythmic ventral root burst activity in response to electrical stimulation of the dorsal surface of the cord or bath applied pharmacological agents (Grillner et al., 1986;

Cangiano and Grillner, 2003; see Soffe, 1991 for similar results in *Xenopus*). In addition, application of strychnine to the spinal cord converted left-right alternating burst activity to synchronous bursts (Cohen and Harris-Warrick, 1984; Hagevik and McClellan, 1994). Computer modeling of these results suggests that left and right oscillators are coupled by relatively strong reciprocal inhibition in parallel with weaker reciprocal excitation (Hagevik and McClellan, 1994). In a second study in which midline lesions usually spanned about half the length of *in vitro* spinal cord preparations from adult lamprey, pharmacologically elicited left-right alternating burst activity was largely abolished in ventral roots in the lesioned part of the spinal cord but was retained in the intact part of the cord (Buchanan, 1999). In separate experiments, photoablation of some CCI's altered the symmetry of left-right bursting (Buchanan and McPherson, 1995). These results were interpreted to mean that the reciprocal inhibition, mediated in part by CCI's, contributes to rhythmogenesis.

3. *Brief Summary of Results*

In the present study, the roles of reciprocal connections between left and right spinal CPG modules in larval lamprey were examined in whole animals and *in vitro* brain/spinal cord preparations with longitudinal midline lesions in the rostral or caudal spinal cord. Instead of activating spinal locomotor networks by bath applied pharmacological agents or by non-specific electrical stimulation of the surface of the spinal cord, motor activity was initiated in a more physiological fashion from the brain with pharmacological stimulation and recorded in both intact and lesioned regions of spinal cord. The results suggest that in the absence of connections with intact regions of cord, isolated left and right hemi-spinal cords are not able to generate locomotor burst

activity in response to descending activation from locomotor command systems in the brain. Thus, in larval lamprey commissural interneurons that couple right and left spinal locomotor networks appear to contribute to left-right phasing of burst activity as well as rhythmogenesis. Parts of this study have been presented in abstract form (Jackson et al., 2003).

Figure 1. The basic components of the locomotor command system in lamprey: (a) a brainstem command system activates the spinal locomotor networks, (b) spinal locomotor oscillators produce the basic pattern, and (c) a coordinating system couples the spinal oscillators to produce a central pattern generator (CPG) and to ensure proper timing of the locomotor activity along the spinal cord. The spinal CPGs activate motoneurons (MNs) that activate the muscle. Mechanosensory input to the CPG can shape the basic pattern.

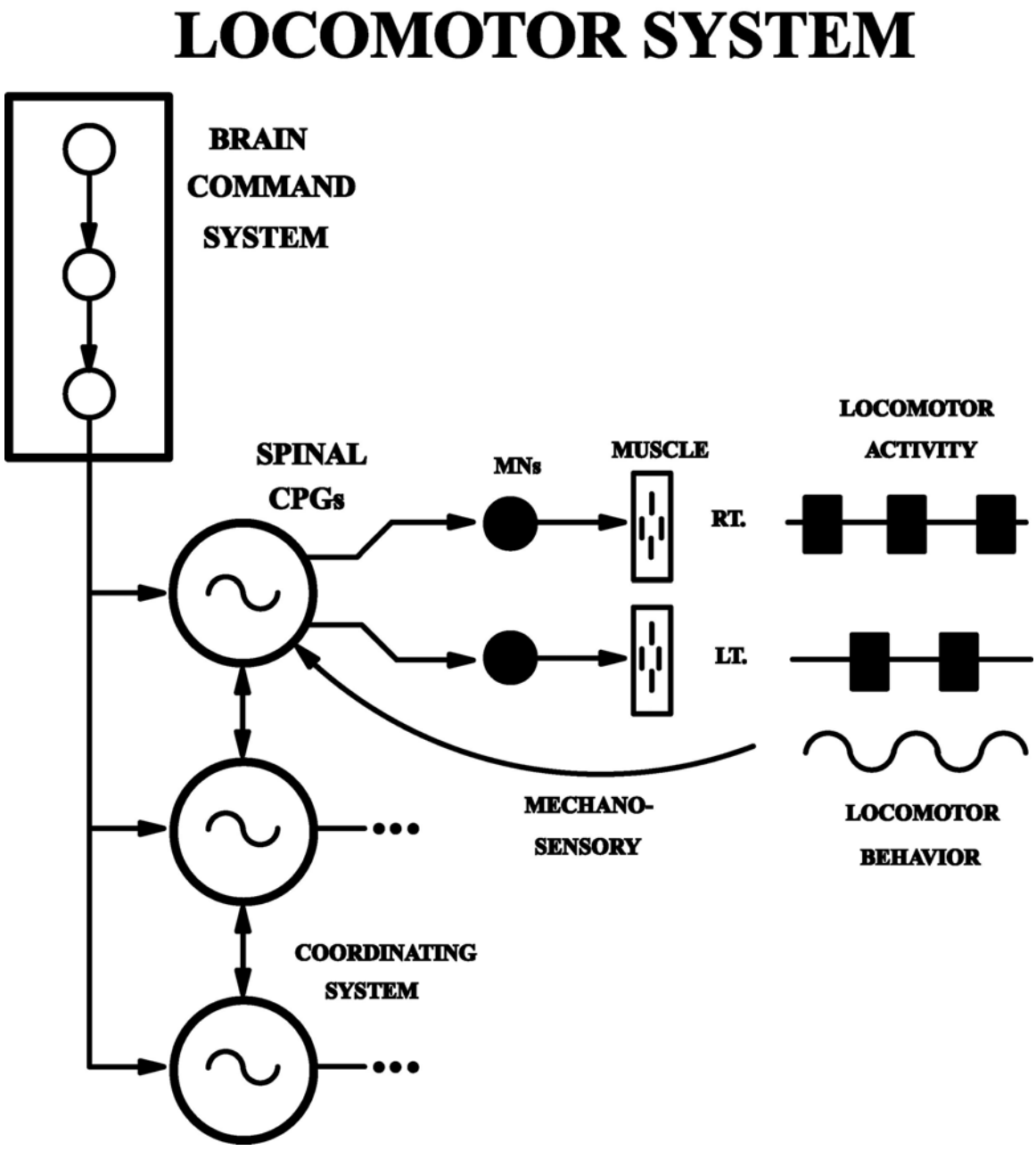
LOCOMOTOR SYSTEM
BRAIN
COMMAND
SYSTEM
SPINAL
CPGs
MNs
MUSCLE
RT.
LT.
LOCOMOTOR
ACTIVITY
MECHANO-
SENSORY
LOCOMOTOR
BEHAVIOR
COORDINATING
SYSTEM

Figure 1

CHAPTER II

METHODS

GENERAL METHODS

1. *Animal Care*

Larval sea lamprey (*Petromyzon marinus*) that were used for whole animal experiments, semi-intact preparations, and *in vitro* brain/spinal cord preparations were maintained in ~10 l aquaria at 23-25°C. The procedures utilized in the present study were approved by the Animal Care and Use Committee at the University of Missouri (protocol 1471).

2. *In Vitro Brain/Spinal Cord Preparations*

In vitro brain/spinal cord preparations were set up as previously described (Hagevik and McClellan, 1994; MClellan, 1994). Briefly, animals were anesthetized, the body below the anus was removed, most of the rostral body musculature surrounding the notochord was removed, and the dorsal surface of the brain and spinal cord were exposed. The preparation was then pinned dorsal side up in a recording chamber containing oxygenated lamprey Ringer's solution (McClellan, 1990a) maintained at 6-9°C. The choroid plexus was removed over the third and fourth ventricles, the cerebellar commissure was transected, and the obex was extended caudally to allow access to the ventricular surface of the brain for pharmacological microstimulation (see below). To eliminate mechanosensory inputs from contractions of the remaining musculature around the cranium and notochord d-tubocurarine chloride (15 mg/l; Sigma Chemical) was added to the bath. In some animals curare has a significant blocking effect on $GABA_A$

receptors (Siebler et al., 1988). However, in the lamprey brain-initiated *in vitro* locomotor activity is virtually identical in the absence of curare or with as much as 150 mg/l (i.e. 10X the concentration used in the present study) applied to the spinal cord (P. Hinton and A.D. McClellan, unpublished data). Suction electrodes were placed in contact with ventral roots to record spinal locomotor activity (1-3 Fig. 5A or 1-4 Fig 25A). In addition, the caudal end of the spinal cord was drawn into a suction electrode (SC, Fig. 5A) to record spinal cord activity under those situations when chemical microstimulation did not initiate ventral root locomotor activity.

3. *Brain Areas used for Pharmacological Microstimulation*

In semi-intact (see below) and *in vitro* brain/spinal cord preparations from larval lamprey, spinal locomotor activity can be elicited by pharmacological microstimulation in at least five brain areas (see Introduction): DLM; VMD; RLR; MLR; and reticular nuclei (ARRN, MRRN, PRRN) (Hagevik et al., 1996; Hinton and McClellan, 1997; Paggett et al., 2000; Sirota et al., 2000) (see Fig. 2B or 4). In the present study, pharmacological microstimulation in the MLR proved to be unreliable, and also the movements elicited by unilateral electrical and pharmacological stimulation in this locomotor region have been partially characterized (Sirota et al., 2000). The difference between this previous study and the present study may be due in part to differences in the methods that were used to activate the MLR. Therefore, the present study focused on responses elicited by stimulation in the RLR, VMD, or DLM locomotor areas as well reticular nuclei. In order to ensure that pharmacological agents applied to these brain areas were acting locally, pharmacological microstimulation also was applied just outside of the effective stimulation areas.

Pharmacological microstimulation is thought to activate cell bodies and dendrites, but not, as a rule, axons of passage (Goodchild et al., 1982). In the present study, bilaterally symmetrical pharmacological microstimulation (PE_R and PE_L, Fig. 2A, 5A) was used to identify brain locomotor areas, which when stimulated, initiated spinal locomotor activity, as previously described in detail (Hagevik and McClellan, 1994b; McClellan, 1994; McClellan and Hagevik, 1997). In addition, this technique was used to determine the effective sizes, pharmacology (i.e. EAA receptors), and organization of higher brain locomotor areas.

4. *Pharmacological Microstimulation*

Pharmacological microstimulation was used, as previously described in detail, to elicit spinal locomotor activity, bouts of swimming, or other movements (Hagevik and McClellan, 1994b; McClellan, 1994; Hagevik et al., 1996; McClellan and Hagevik, 1997). Two micropipettes were filled with 5 mM D-glutamate and 5 mM D-aspartate in lamprey Ringer's solution (pH 7.2-7.4), and Fast green was added to visualize the ejection bolus. The above concentrations are about five times those that are typically used for bath application of these agents in lamprey experiments (Grillner et al., 1981; Hagevik and McClellan, 1994a; Rovainen, 1985). Since agent concentration decreases rapidly with distance from the tip of a pressure ejection micropipette (Stone, 1985), the average concentrations at the stimulation sites probably were much less and within the physiological range. However, it should be emphasized that even with pressure ejection in a homogenous medium, it is very difficult to accurately estimate concentration versus distance from the stimulating micropipette at various times (Stone, 1985; see Discussion). The tips of the micropipettes were broken off (~2-5 μm tip size) and positioned

bilaterally and symmetrically in one of three brain locomotor areas (Paggett et al., 2004): ventromedial diencephalon (VMD); dorsolateral rhombencephalon (DLM); and rostrolateral rhombencephalon (RLR, see Fig. 2B, 4A,B, 5B), or reticular nuclei. The tips of the micropipettes were inserted about 25-50 μm below the ventricular surface (Fig. 2A,5A) where most neuronal cell bodies, including RS neurons, are located (Niewenhuys, 1977; Ronan, 1989). The amount of excitatory agent ejected from each micropipette was adjusted by varying the duration of the applied pressure pulses (7-20 ms pulses delivered at 1 Hz; ~20 psi, same pressure applied to both pipettes) (Sakai et al., 1979; Palmer, 1982; Stone, 1985). In general, a single pressure pulse ejected a bolus with a diameter of ~8-16 μm (~0.256-2.0 pl). At the end of a stimulation sequence (usually < 1 min), the radius of the ejection area within the tissue that was stained with Fast green was less than ~50-75 μm (width of brain ~1 mm). Following each stimulation sequence, a period of at least 3 minutes was allowed before stimulation was performed again in the same locomotor area.

LOCOMOTOR MOVEMENTS INITIATED BY PHARMACOLOGICAL MICROSTIMULATION IN HIGHER LOCOMOTOR COMMAND AREAS (CH. III)

1. *Semi-Intact Preparation*

Lamprey (83-119 mm, n = 33 animals) were anesthetized in tricaine methanesulphonate (MS222, ~200mg/l; Sigma, St. Louis, MO), and a ventral midline incision was made from the last gill to the cloaca. The animals were then eviscerated and pinned dorsal side up in a dissection dish. Approximately the rostral quarter of the body

musculature and remaining tissue around the notochord were removed, and the dorsal surface of the brain and spinal cord were exposed, similar to the procedure for *in vitro* brain/spinal cord preparations (Hagevik and McClellan, 1994; McClellan, 1994). The preparations were then pinned dorsal side up in a recording dish (63 x 172 mm) containing oxygenated Ringer's solution (McClellan, 1990) maintained at 4-10^{o}C. A Vaseline-sealed Plexiglass barrier was placed a short distance caudal to the brain (segment 18.5 ± 5.5) to create a brain pool (Pool I) and a caudal pool in which the lower part of the body could move freely (Pool II) (see Fig. 2A). Usually, 15 mg/l D-tubocurarine chloride (Sigma; St. Louis, MO) was added to Pool I to prevent movements resulting from contraction of musculature in the head. Typically, episodes of locomotor movements could be initiated by sensory stimulation, usually a brief tail pinch, within 1-2 hours after the animal was removed from anesthetic and placed in the recording dish.

2. *Pharmacological Microstimulation*

In semi-intact preparations, for stimulation in higher order locomotor areas (VMD, RLR, DLM) (n = 26), the brain pool (Pool I, Fig. 2A) contained Ringer's solution. For stimulation in reticular nuclei (n = 13), a low-calcium Ringer's solution containing 0.26 mM $CaCl_2$ and 2 mM $MnCl_2$ (10 mM PIPES was substituted for HEPES; pH = 7.4) was added to the brain pool (Pool I) to block chemical synaptic transmission (McClellan, 1984). Since it was not possible to activate all RS neurons in a reticular nucleus and simultaneously prevent the spread of the pharmacological agent outside the nucleus, reticular nuclei were subdivided into smaller areas for the purposes of microstimulation (see Fig. 2B). However, in the ARRN only stimulation in the anterior division (aARRN) and in the MRRN only stimulation in the posterior division (pMRRN)

was effective in initiating locomotor movements and muscle activity (see Fig. 2B, Tables 1-3). In contrast, in the PRRN, stimulation in either the anterior, middle, or posterior areas were effective, and since these three areas are in the same nucleus and produced similar results, the data were pooled (see Fig. 2B, Tables 1-3).

Following pharmacological microstimulation in the above brain locomotor areas, the stimulation sites were marked with ejection of a small amount of ~1% Alcian blue (Sigma; St. Louis, MO) in Ringer's solution, as described previously (Paggett et al. 2004). These marked stimulation sites persisted during subsequent histological processing (see below).

3. *Histological Processing*

To confirm that certain Alcian-marked stimulation sites were, in fact, within reticular nuclei (see Fig. 13,14), for semi-intact experiments in which stimulation was applied to these areas of the brain, descending brain neurons were retrogradely labeled with horseradish peroxidase (HRP) (n = 10). The spinal cord was transected at ~15-20% body length (BL, relative distance from the head), and ~5 mm of the caudal end of the spinal cord was gently drawn up into a fire-polished glass suction electrode. The liquid in the electrode was removed and replaced with a solution containing 40% HRP and 1% dimethyl sulphoxide (DMSO), after which the HRP was allowed to transport for 36-48 hours. Subsequently, the brain and rostral spinal cord were removed and processed histologically for HRP using a modified Hanker-Yates protocol, as previously described in detail (Davis and McClellan, 1994; Zhang et al., 2002). Following histological processing for HRP, whole-mount brains were dehydrated in an ethanol series, cleared in methyl salicylate, placed on slides, and coverslipped with Permount, as previously

described (Davis and McClellan, 1994). For experiments in which pharmacological stimulation was only applied to higher order locomotor areas, HRP was not applied to the spinal cords, and the brains were histologically processed without the HRP reaction step. Using a custom computer marking/tracing system, the outlines of the brains were traced, stimulation sites were marked, and, if applicable, outlines were traced around reticular nuclei delineated by HRP-labeled RS neurons (see Fig. 13,14).

4. *Kinematic Analysis*

During anguilliform type swimming in lamprey and other slender fish, the head displays significant left-right lateral displacement, and the amplitudes of lateral displacement increase gradually from the head to the tail (Grillner and Kashin, 1976). In the present study, the amplitudes of lateral displacement along the body during undulatory movements initiated by stimulation in brain locomotor areas in semi-intact preparations were compared to those during swimming in whole animals (Davis et al., 1993).

Whole animals (91-103 mm, n = 6 animals) were placed in a longitudinal swim tank (8 x 74 cm) and videotaped with an S-VHS camera (Panasonic PVS 770; Yokohama, Japan; 30 frames/s, 8 ms shutter speed;) that was 1334 mm above the animals. Bouts of swimming were evoked by either tactile stimulation or brief electrical stimulation (1-10 mA, 2-ms pulses at 100 Hz for 50 ms) applied to the oral hood or tail. Episodes of swimming movements were then played back into a computer, and video frames were captured using an image-capturing device (Dazzle Digital Photo Maker, SCM Microsystems, Fremont, CA). The captured frames were analyzed with custom image digitizing software. For each frame, eleven x,y coordinates were marked along the

body, including the head, tail, and the points of maximal lateral displacement (see Fig. 3A) (see Davis et al., 1993), and the sets of points defining the body in each frame were imported into a spreadsheet (Lotus 1-2-3). For a single video frame in each episode, the distance between the points of maximal lateral displacement (l) and animal length (L) were used to calculate the number of wavelengths along the body ($\lambda = 2l/L$ body lengths per cycle; Table 2) (Williams et al., 1989). In addition, the wavelength was then used to calculate the mechanical phase lag ($\phi = 0.5/$[segments between points of max. lateral displacement]; where the segment number at a particular percent body length was calculated from an empirically derived equation; segment = [1.2 * %BL - 10]; Table 2). Cycle times (T) for swimming movements were calculated as the number of frames encompassing a full cycle times the interframe interval (= 33.3 ms) (Table 2).

For semi-intact preparations, kinematic parameters were calculated from preparations that did not have muscle recording electrodes (n = 15). Pharmacological stimulation in specific brain areas (see above) elicited locomotor movements and other responses that were video taped with an S-VHS video camera (Panasonic KP-2222; Yokohama, Japan; 30 frames/s) that was 483 mm above the preparations. Coordinates along the body were determined as described above for swimming in whole animals and imported into a spreadsheet, which performed the following mathematical manipulations for each episode, as described previously (Davis et al., 1993): (1) calculated the axis of swimming using multiple linear regression analysis from all of the coordinates, and centered the frames on the y-axis (Fig. 3C); and (2) rotated the axis of swimming to the x-axis and calculated new body position coordinates. The points of maximal lateral displacement versus distance from the head were extracted from each frame and

normalized to body length. The normalized maximal amplitudes of lateral displacement were plotted versus the normalized distance from the head (Fig. 3D). In addition, cycle times, wavelengths, and mechanical phase lags for episodes of swimming movements in semi-intact preparations were determined in the same manner as described above for whole animals.

5. *Muscle Activity*

In some semi-intact preparations (n = 23), pairs of fine copper wires (60 μm diam.), insulated except at the tips, were inserted into body musculature at ~40-50% BL (electrodes 1 and 2; Fig. 2A) or ~60-70% BL (electrode 3; Fig. 2A) to record muscle activity (EMGs) (Fig. 2C). Locomotor movements were initiated by pharmacological microstimulation in brain locomotor areas and videotaped, while muscle activity was simultaneously recorded, amplified by 1000X, filtered (100 Hz-5 kHz), and stored on tape (Neuro-Data DR890; Cygnus Technologies, Delaware Water Gap, PA; 11 kHz sampling rate per channel). Video frames were indexed electronically in time so they could be synchronized with muscle recordings (McClellan, 1990). Following muscle recordings, the numbers of body segments between ipsilateral recording electrodes were counted.

Episodes of brain-initiated muscle activity were captured with custom data acquisition and analysis software. Subsequently, for episodes of symmetrical locomotor movements that had relatively constant cycle times, the onsets and offsets of muscle burst activity were marked. These data were imported into a spreadsheet program (Lotus 1-2-3) that calculated and graphed parameters of locomotor activity (Table 3): (a) cycle time (T) was the interval between the midpoints of successive cycles; (b) burst proportion

(BP) was calculated as the burst duration divided by cycle time; (c) intersegmental phase lag (ϕ_{INT}) was calculated as the ratio of delay between the midpoints of ipsilateral bursts and cycle time divided by the intervening numbers of segments; and (d) right-left phase value (ϕ_{RT-LT}) was the phase of the midpoints of right bursts within cycles defined by the midpoints of left bursts.

6. *Statistics*

For whole animals and semi-intact preparations, the average wavelengths were compared to 1.0 using a Student's t-test (Table 2). For semi-intact preparations, the average values for cycle time, wavelength, and mechanical phase lag for swimming movements initiated from the various stimulation sites were compared to the corresponding values for swimming in whole animals using one-way ANOVA (Table 2). Finally, for semi-intact preparations, the parameters of muscle burst activity initiated by pharmacological microstimulation in different brain areas (see above) were compared to those for muscle activity in whole animals (Davis et al., 1993; Paggett et al., 1998) using either a Student's t-test or one-way ANOVA (Table 3). Statistical significance was considered for $P \leq 0.05$.

SIZE, PHARMACOLOGY, AND ORGANIZATION OF HIGHER LOCOMOTOR COMMAND AREAS IN LARVAL LAMPREY (CH. IV)

1. *Retrograde Labeling with HRP*

During *in vitro* experiments, stimulation sites in the brain were mapped and marked with a dye (see below, Fig. 4A). In order to provide anatomical references for these marked stimulation sites, prior to *in vitro* mapping experiments, descending brain

neurons, ~80% of which are reticulospinal (RS) neurons (Davis and McClellan, 1994a), and descending trigeminal tracts were pre-labeled with horseradish peroxidase (HRP) (see Fig. 4, 20A). Approximately 10-14 days prior to setting up *in vitro* preparations from larval lamprey (n = 23, 100-140 mm), animals were anesthetized in tricaine methanesulphonate (MS222, ~200 mg/l; Sigma Chemical, St. Louis, MO). The spinal cord was exposed and transected at 60% body length (BL, relative distance from head), and a Gelfoam pledget (Upjohn; Kalamazoo, MI) soaked in a 40% HRP (Type VI; Sigma Chemical, St. Louis, MO) and 1% dimethyl sulphoxide solution was applied to the spinal cord, as previously described (Davis and McClellan, 1994; Zhang et al., 2002). Trigeminal sensory inputs can elicit locomotor activity (McClellan, 1984), and these afferents synapse with second order sensory neurons in the nucleus of the descending trigeminal tract (Northcutt, 1979) that presumably have inputs to brain locomotor areas. Therefore, for larval lamprey in which stimulation was performed in the rhombencephalon (n = 13, 105-140 mm), ~3-5 days prior to setting up *in vitro* preparations, sensory axons in the descending trigeminal tracts (dV), as well as trigeminal motor neurons, were pre-labeled (Fig. 4B; note that trigeminal motoneurons were omitted for clarity). A ~3-4 mm transverse incision was made between the anterior tip of the oral hood and the naris, and a small Gelfoam pledget soaked in the HRP solution above was placed in the incision site, as previously described (Calton et al., 1998). Following *in vitro* experiments, brains were processed for HRP (see below).

2. *In Vitro Brain/Spinal Cord Preparations*

In vitro preparations from larval lamprey (n = 37, 93-140 mm) were set up as described in detail (see above). In several preparations (n = 11, 93-127 mm), a Vaseline-

sealed barrier was placed at segment ~5-12 to create a brain pool (Pool I) and a spinal cord pool (Pool II) (dark vertical line, Fig. 22A).

3. *Brain Locomotor Areas and Pharmacological Microstimulation*

In order to determine the sizes of the above brain locomotor areas, the tips of the stimulation pipettes were systematically moved in and around the areas. Since very different responses could be elicited from stimulation sites that were relatively close (~50 μm), substantial diffusion of stimulating agents and activation of neurons that were great distances from the stimulation site were unlikely. Following stimulation at a particular site in the brain, a separate manipulator was used to position the tip of a different micropipette containing 1-3% Alcian blue (Sigma Chemical) in Ringer's solution at the site. A small amount of Alcian blue was pressure ejected just below the dorsal surface of the brain to bilaterally mark the stimulation sites (Harnishchfeger, 1979). Alcian blue did not appear to compromise the viability or responsiveness of *in vitro* preparations and was stable during subsequent histological processing (see below). In addition, this method was preferable to marking stimulation sites with electrolytic lesions, which might compromise preparations and affect the responses elicited by subsequent pharmacological microstimulation. After histological processing (see below), each Alcian blue mark in the tissue was matched with the pharmacological agents that were tested at the stimulation site and the quality of initiated locomotor activity. After testing one stimulation site in the brain, the tips of the micropipettes were moved ~50 μm to an adjacent microstimulation site, and the procedures described above were repeated. The stimulation sites were confined to within a given brain locomotor area that was effective in initiating locomotor activity (see below) and a circumscribed region around the

locomotor area that was ineffective (i.e. elicited uncoordinated ventral root activity or did not elicit activity; see Fig. 16B). Anatomical structures, such as the infundibulum, mesencephalic-rhombencephalic border, or the sulcus limitans, sometimes prevented testing of all the potential sites surrounding locomotor areas (see Figs. 16B,18B,20B).

Once the brain locomotor areas had been located using D-glutamate/D-aspartate, the pharmacology of the brain areas was determined. Specifically, the subtypes of excitatory amino acid (EAA) receptors present in a locomotor area were determined by pressure ejection of one or more of the following agents: N-methyl D-aspartate (NMDA, 0.5 mM); kainate (KA, 0.25 mM); or α-amino-3-hydroxy-5-methyl-isoxazole-4-propionic acid (AMPA, 1 mM) (all from Sigma Chemical). These agents were dissolved in lamprey Ringer's solution (pH = 7.2 - 7.4), and Fast Green was added to visualize the ejection bolus. As was the case for 5 mM D-glutamate/5 mM aspartate, the concentrations for NMDA and KA are approximately five times the typical bath concentrations that have been used in the lamprey (Grillner et al., 1981; Rovainen, 1985), while the concentration for AMPA was taken from previous pressure ejection studies (Alford and Grillner, 1990; El Manira et al., 1997; Krieger et al., 1998; Sirota et al., 2000). In the lamprey, all three subtypes of ionotropic glutamate receptors have been found on RS neurons (Dryer, 1988; Zompa and Dubuc, 1998). A small amount of Alcian blue was pressure ejected to bilaterally mark each of the stimulation sites.

4. *Organization of Locomotor Command System*

Results from a previous study suggest that neurons in the RLR project to locomotor areas in the DLM and VMD, and these latter areas project to RS neurons, which initiate spinal locomotor activity (Paggett et al., 2004). In the present study, two

types of experiments were performed to examine further the organization of parts of the locomotor command system. First, for certain *in vitro* experiments (n = 11, 93-127 mm), a Vaseline-sealed barrier was placed at segment ~5-12 to create a brain pool (Pool I) and a spinal cord pool (Pool II) (dark vertical line, Fig. 22A). Suction electrodes were placed in contact with ventral roots to record spinal locomotor activity (1,2 at segment 16-27, ~22-31% BL; 3 at segment 40-49, ~42-49% BL; Fig. 22A). In addition, the caudal end of the spinal cord was drawn into a suction electrode (SC, at segment 58-83, ~57-78% BL; Fig. 22A) to record spinal cord activity. Well-coordinated spinal locomotor activity was elicited by pressure ejection of 5 mM D-glutamate/5 mM D-aspartate in higher order locomotor areas, specifically the VMD, DLM, or RLR. Subsequently, synaptic transmission was blocked in the brain by application of a zero calcium Ringer's solution to the brain pool (Pool I), and pharmacological stimulation was applied to the same locomotor areas (n = 11). The purpose of these experiments was to test whether these higher order locomotor areas could directly activate spinal locomotor networks or whether a relay through other brain neurons was required.

Second, in a different set of experiments (n = 3), pharmacological microstimulation in the RLR with 5 mM D-glutamate/5 mM D-aspartate was used to initiate well-coordinated spinal locomotor activity. Subsequently, the stimulation pipettes were withdrawn, and a complete transection was made at the mesencephalic-rhombencephalic border with iridectomy scissors. Stimulation in the RLR was repeated to test whether spinal locomotor activity could be initiated entirely via neural circuits in the rhombencephalon or whether more rostral circuitry (e.g. VMD, DLM) was required. For example, it has been suggested that trigeminal sensory inputs initiate locomotion by

activation of RS neurons via a disynaptic pathway that is entirely within the rhombencephalon (Viana Di Prisco et al., 1997,2005; LeRay et al., 2004). However, previous studies from our laboratory suggest that neurons in the RLR, which may receive inputs from trigeminal second order sensory neurons in the rhombencephalon (Paggett, 1999), project to locomotor areas in the VMD and DLM, which then activate RS neurons (Paggett et al., 2004).

5. *Data Recording and Analysis*

Ventral root activity was rectified and integrated (τ = 50 ms) to better reveal locomotor bursts. Brain-initiated locomotor and spinal cord activity (Fig. 5C1-3) was acquired using custom data acquisition and analysis software. Five criteria were used to determine whether a chemical microstimulation site was considered "effective" in initiating good quality locomotor activity (see Fig. 5, Tables 4,5): (a) stimulation with a given agent had to initiate at least ten cycles of locomotor activity, but almost all locomotor episodes included more cycles; (b) locomotor activity had to be elicited within 1 min of the onset of pharmacological stimulation, although much shorter latencies were typical (see Tables 4,5); (c) *in vitro* spinal locomotor activity had to consist of left-right alternation of burst activity at the same segmental level ($1 \leftrightarrow 2$) and a rostrocaudal phase lag of ipsilateral locomotor burst activity ($2 \rightarrow 3$) (see below and Fig. 5); (d) the cycle times (T, see below) of locomotor activity had to be less than 3 s; and (e) stimulation at a given site in the brain had to initiate locomotor activity during at least two consecutive trials. With the data acquisition system, the offsets and onsets of locomotor burst activity were marked and imported into a spreadsheet program for the calculation of locomotor parameters (McClellan and Hagevik, 1997): cycle times (T) were defined as the interval

between the onsets of locomotor burst activity in successive cycles; burst proportions (BPs) were calculated as the duration of burst activity divided by cycle time; intersegmental phase lags (ϕ_{INT}) were defined as the ratio of the delay between the midpoints of ipsilateral locomotor burst activity and cycle time, divided by the number of intervening segments; and right-left phase values (ϕ_{RT-LT}) were calculated as the phase of the midpoints of right locomotor burst activity within cycles defined by the midpoint of left locomotor burst activity.

6. *Histological Processing*

For those *in vitro* preparations in which HRP was applied prior to the experiments, the brains and spinal cords were removed and processed for HRP using a modification of the Hanker-Yates protocol, as previously described (Davis and McClellan, 1994; Zhang et al., 2002). After the tissue was dehydrated in an ethanol series and cleared in methyl salicylate, whole mounts were prepared on slides with Permount (Fisher Scientific; Fair Lawn, NJ). Subsequently, a custom computer-based microscope marking/tracing system was used to trace around the outlines of the brains, reticular nuclei, and descending trigeminal tracts (dV) (see Fig. 4A,B). Labeled descending brain neurons could easily be distinguished from the tightly clustered trigeminal motoneurons (Calton et al., 1998), which were relatively uniform in size and shape, very darkly stained, and in a slightly different focal plane. Finally, the Alcian blue-labeled stimulation spots were marked (see Fig. 4). The marking/tracing system produced a two-dimensional plot of the brain as if all structures, HRP-labeled neural elements, and marked pharmacological microstimulation sites were in a single plane of focus (see Figs. 4, 5B, 16A,B). Outlines of reticular nuclei and descending trigeminal

tracts provided reference points and "landmarks" so that diagrams of effective stimulation sites could be made and compared (see Figs. 16,17 for VMD locomotor area). Note that because the whole mount brains were flat, ventral (dorsal) areas of the diencephalon and mesencephalon appear lateral (medial) in the tissue (e.g. Fig. 4).

7. *Statistics*

For each brain locomotor area (RLR, DLM, VMD) in each preparation, the sites that appeared to initiate the most stable locomotor activity with least variation of cycle times were defined as the "best sites". The parameters of spinal locomotor activity for the "best site" for each brain locomotor area and for each pharmacological agent (e.g. Fig. 16C1,18C1,20C1) were averaged and compared using one-way ANOVA (Tables 4,5). Values were considered to be statistically significant for $P \leq 0.05$.

DISRUPTION OF LEFT-RIGHT RECIPROCAL CONNECTIONS IN THE SPINAL CORD OF LARVAL LAMPREY (CH. V)

1. *Whole Animals*

Midline spinal cord lesions. Animals (112-157 mm, n = 38) were anesthetized in MS222, as previously described, transferred to a dissection dish, and pinned dorsal side up (i.e. a pin through the oral hood and another pin through the caudal tail). Gauze moistened with lamprey Ringer's solution (McClellan, 1990a) was placed over the animal except in the area where the spinal cord lesions were made, and ice chips were placed on the gauze to cool the animal and reduce bleeding during surgery. Except where specifically stated, all spinal cord lesions were made in the evenings, and after a 1-2 day recovery period, preparations were set up for EMG recordings.

A longitudinal incision was made along the dorsal midline at one of three different regions of the body (see following text), and the spinal cord and overlying meninges were exposed. With a fine scalpel blade (Beaver "mini-blade" #376500; Arista Surgical Supply, New York, NY), one of the following three types of longitudinal midline lesions of the spinal cord were made to interrupt coupling between left and right spinal locomotor networks: (a) "short" caudal lesion, continuous lesion extending from 35%→45% body length (BL, normalized distance from the head) (data not shown; n = 12); (b) "long" caudal lesion, continuous lesion extending from 30%→50% BL (Fig. 24A; n = 12); and (c) "long" rostral lesion, continuous lesion extending from 8%→30% BL (Fig. 28A,29A; n = 16). The completeness of the longitudinal lesions was verified by gently displacing the hemi-spinal cords laterally. It should be noted that in larval lamprey, the midline of the spinal cord is readily visible under the dissecting microscope. In about half the animals in each of the above three groups, a complete spinal cord transection was made with iridectomy scissors at the caudal end of the midline spinal lesion to eliminate ascending inputs from more caudal spinal neural networks. The edges of the incision were manually pinched together and sealed with several very small, evenly spaced drops of cyanoacrylate (Super Glue Gel, Loctite Co.; Rocky Hill, CT). Since animals with caudal midline spinal cord lesions were able to generate caudal muscle burst activity, it is unlikely that glue diffused into the incision and affected spinal circuitry. Subsequently, animals were placed in a tank that was bubbled with oxygen for ~1 hour and then transferred to their home aquariums to recover for ~1-2 days before locomotor movements and muscle activity were recorded. In general, the behavioral capabilities of

lesioned animals were similar immediately after recovery from anesthesia and 1-2 days later when muscle recordings were performed.

Locomotor movements and muscle activity. Prior to inserting muscle recording electrodes, animal movements were videotaped from overhead with an S-VHS camera (Panasonic PVS 770; 30 frames/s, 1/125 s shutter speed) to document the behavioral capabilities of each animal. Locomotor and/or flexure responses were evoked by tactile stimulation or brief electrical stimulation (1-10 mA, 2 ms pulses at 100 Hz for 50 ms) applied to the oral hood (anterior head) or tail. Subsequently, animals were anesthetized, and pairs of copper wires (see above), were inserted into body musculature at ~20% BL (electrodes 1 and 2) and ~40% BL (electrodes 3 and 4; see Figs. 24A, 28A, and 29A) to record muscle activity (EMGs). Locomotor and/or flexure movements were videotaped, and simultaneously muscle activity was recorded, amplified (1000X), filtered (100 Hz – 5 kHz), and stored on tape (Neuro-Data DR890, Cygnus Technologies, Delaware Water Gap, PA; 11 kHz sampling rate per channel). Video frames were electronically indexed in time so that they could by synchronized with muscle activity data (McClellan, 1990a). Following muscle recordings, animals were re-anesthetized, and the numbers of body segments between ipsilateral recording electrodes were counted and used for calculating locomotor parameters (see following text).

2. *In Vitro Brain/Spinal Cord Preparations*

Larval sea lamprey (104-135 mm, n = 24) were anesthetized, and *in vitro* preparations were set up, as previously described (Hagevik and McClellan, 1994; McClellan, 1994, see above). *In vitro* brain/spinal cord preparations (Fig. 25A) were used to investigate whether locomotor activity could be elicited in hemi-spinal cord

regions in the absence of mechanosensory inputs. In some preparations, Vaseline-sealed plastic barriers were used to create a brain pool (Pool I) and one or two spinal pools (Pool II, Figs. 31A and 32A; Pools II and III, Fig. 26A). A low-calcium Ringer's solution, which included 10% normal calcium and 2 mM $MnCl_2$ (McClellan, 1984), was used to block synaptic transmission in restricted regions of the spinal cord. Unless otherwise stated, suction electrodes (1-4, Fig. 25A) were placed in contact with ventral roots at ~20% BL and ~40% BL to record spinal motor activity. In general, *in vitro* dissections were performed in the evenings, and on the following day (i.e. first day) recordings were made before and during a period 15-240 min after performing spinal cord lesions. Recordings were not made on the second day because the in vitro preparations can become less responsive and less excitable during this time.

Pharmacological microstimulation. Spinal motor activity was initiated by pharmacological microstimulation (sometimes referred to as "chemical microstimulation") in one of three brain locomotor areas: VMD, DLM, or RLR (Hagevik and McClellan, 1994; McClellan, 1994; McClellan and Hagevik, 1997; Paggett et al., 2004, see above). Since there were no obvious differences in the results for locomotor activity elicited from these brain locomotor areas, the data from the three areas were pooled. The pharmacological agents were pressure ejected into brain locomotor areas, and evoked ventral root activity was amplified (1000X), filtered (10 Hz–2 kHz), and stored on videotape (NeuroData DR890). For display purposes and data analysis, ventral root activity was rectified and integrated (τ = 50 ms) to better reveal the onsets and offsets of bursts. In some *in vitro* experiments (see Results), following activation of motor activity from the brain, the very rostral spinal cord was transected, and 1 mM D-

glutamate was applied to the spinal cord to elicit ventral root burst activity (Fig. 26B4; see McClellan, 1990b).

Midline spinal cord lesions. First, prior to performing spinal cord lesions, pharmacological microstimulation was applied to brain locomotor areas, and control *in vitro* spinal locomotor activity was recorded (Fig. 25B). Second, in most experiments, "sodium free" choline Ringer's solution was added to the recording chamber to block action potentials while making one of the following midline lesions in the spinal cord with a fine scalpel blade (see above): (a) "caudal" midline lesion (30%→50% BL; Fig. 25A; n = 13); or (b) "rostral" midline lesion (8%→30% BL; Fig. 29A; n = 10). Subsequently, pharmacological microstimulation was applied again to the same brain locomotor area to initiate spinal motor activity. Third, in most experiments, a spinal cord transection was made at the caudal end of the midline spinal lesion to eliminate ascending inputs from more caudal spinal neural networks, and motor activity was then initiated from the brain (Figs. 25 and 29). In some preparations with midline lesions in the rostral spinal cord, recordings were made with suction electrodes from left and right fascicles at the caudal ends of the hemi-spinal cords (Fig. 32A; n = 4).

3. *Data Analysis*

Motor activity from both whole animals and *in vitro* brain/spinal cord preparations was acquired using custom data acquisition and analysis software. For whole animals, episodes of muscle burst activity during relatively straight swimming-like movements were selected for analysis. For certain types of midline spinal cord lesions (see Results), animals did not generate sufficient propulsive force to result in significant forward progression, and in these cases, episodes of rhythmic muscle activity were

analyzed in which undulatory body movements most closely resembled swimming movements. For *in vitro* preparations, episodes of rhythmic motor activity were analyzed in which the motor pattern had reached a steady state and the rhythm frequency was relatively constant (Fig. 25B1; see Fig. 1 in Paggett et al., 2004).

For whole animals and *in vitro* preparations, the onsets and offsets of burst activity were marked and imported into a spreadsheet program for calculating and graphing locomotor parameters. Cycle times (T) were measured as the interval between the onsets of burst activity in successive cycles. Burst proportions (BP) were calculated as the duration of burst activity (onset-to-offset) divided by the cycle time. Intersegmental phase lags (ϕ_{INT}) were defined as the ratio of the delay between the midpoints of ipsilateral bursts and cycle time, divided by the intervening number of segments. Right-left phase values (ϕ_{RT-LT}) were calculated as the phase of the midpoints of right bursts within cycles defined by the midpoints of left burst activity.

In larval lamprey, swimming motor activity in whole animals is characterized by cycle times of ~200-800 ms, while in *in vitro* brain/spinal cord preparations, swimming activity initiated by pharmacological microstimulation in brain locomotor areas has cycle times of ~400-3000 ms (Davis et al., 1993; McClellan, 1994; Paggett et al., 1998; Boyd and McClellan, 2002). In the present study, EMG or *in vitro* burst activity was considered to correspond to swimming behavior if it had the following features: 1) intersegmental phase lags and burst proportions that were not significantly different than those for control locomotor activity; 2) intersegmental phase lags that were significantly different than zero; 3) repeatable in at least two episodes; 4) cycle-to-cycle variations in cycle times that were typical for normal swimming activity and did not vary by more than

an absolute value of 8.1% ± 7.3% (normal whole animals, n = 999 cycles; data analyzed from Davis et al., 1993) or 7.3% ± 6.9% (*in vitro* preparations, n = 463 cycles; data analyzed from present study); and (d) rhythmic activity that had sufficient signal-to-noise ratio so that the onsets and offsets of bursts were clearly visible. In addition to the above criteria, rhythmic EMG and *in vitro* burst activity had to have cycle times within the above respective ranges to be considered representative of swimming behavior.

4. *Statistics*

For whole animals (EMGs) and *in vitro* preparations, the parameters of rhythmic burst activity recorded after various midline spinal lesions and spinal cord transections were compared to those for control locomotor activity using either a Student's t-test or one way ANOVA (Tables 7,8). In addition, intersegmental phase lags were compared to zero (0) with a Student's t-test. Values were considered to be statistically significant for $p \leq 0.05$.

Figure 2. (A) Diagram of semi-intact preparation with Vaseline-sealed barrier (dark, vertical line) separating a brain pool (Pool I) and a caudal pool (Pool II), pharmacological microstimulation pipettes (PE_R and PE_L), and muscle recording electrodes (1, 2 and 3; see Methods). (B) Idealized brain showing contours around reticular nuclei (mesencephalic reticular nucleus [MRN], anterior rhombencephalic reticular nucleus [ARRN], middle rhombencephalic reticular nucleus [MRRN], and posterior rhombencephalic reticular nucleus [PRRN]). Pharmacological microstimulation sites are shown in the ventromedial diencephalon (VMD, open squares), dorsolateral mesencephalon (DLM, open circles), and rostrolateral rhombecephalon (RLR, open triangles). In reticular nuclei, stimulation sites were located in the anterior and posterior ARRN (aARRN, pARRN), in the anterior and posterior MRRN (aMRRN, pMRRN), and three areas within the PRRN (filled circles). (C) Muscle burst activity recorded from electrodes at 47% body length (BL, normalized distance from the anterior head) showing left-right alternation ($1 \leftrightarrow 2$) of muscle burst activity elicited by bilateral pharmacological microstimulation (PE = pressure ejection pulses) in the RLR (see Methods).

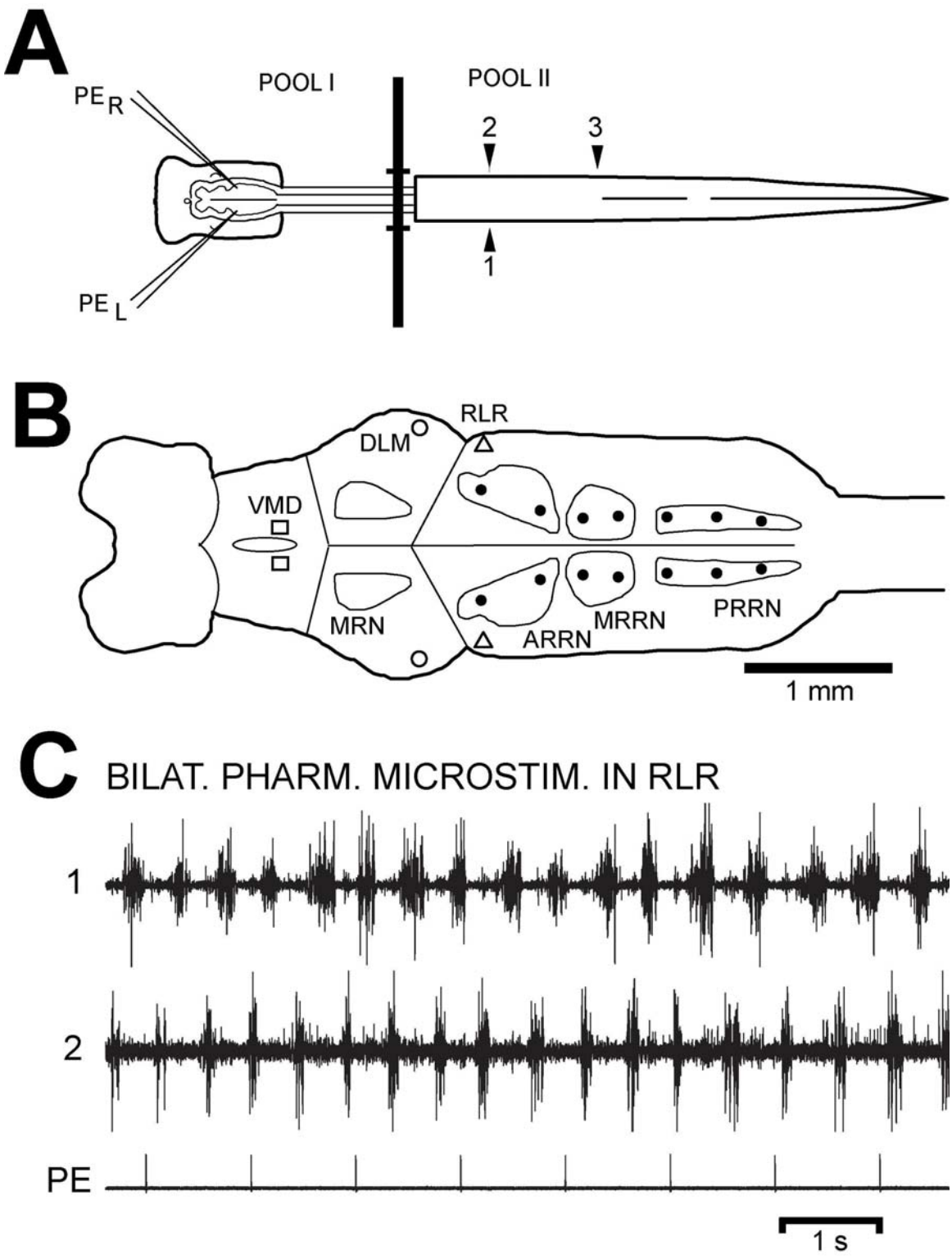
A
POOL I
POOL II
PE R
PE L
1
2
3
B
RLR
DLM
VMD
MRN
ARRN
MRRN
PRRN
1 mm
C
BILAT. PHARM. MICROSTIM. IN RLR
1
2
PE
1 s

Figure 2

Figure 3. (A) Simulated single video frame of the body during swimming. The lateral movements of the body increase with increasing distance from the head, as indicated by the envelope of maximum lateral displacement (dashed lines) and the locations of maximum lateral displacement for this particular frame (arrows). (B) Schematic diagram from an actual semi-intact preparation showing sequential body movements, left to right, elicited by bilateral pharmacological microstimulation in the RLR (cycle time [T] = 990 ms and interframe interval [IFI] = 100 ms). (C) Sequential, superimposed body movements taken from B showing symmetrical movement about the midline (vertical line). (D) Plot of maximum normalized lateral displacement versus normalized distance from the head, taken from B and C.

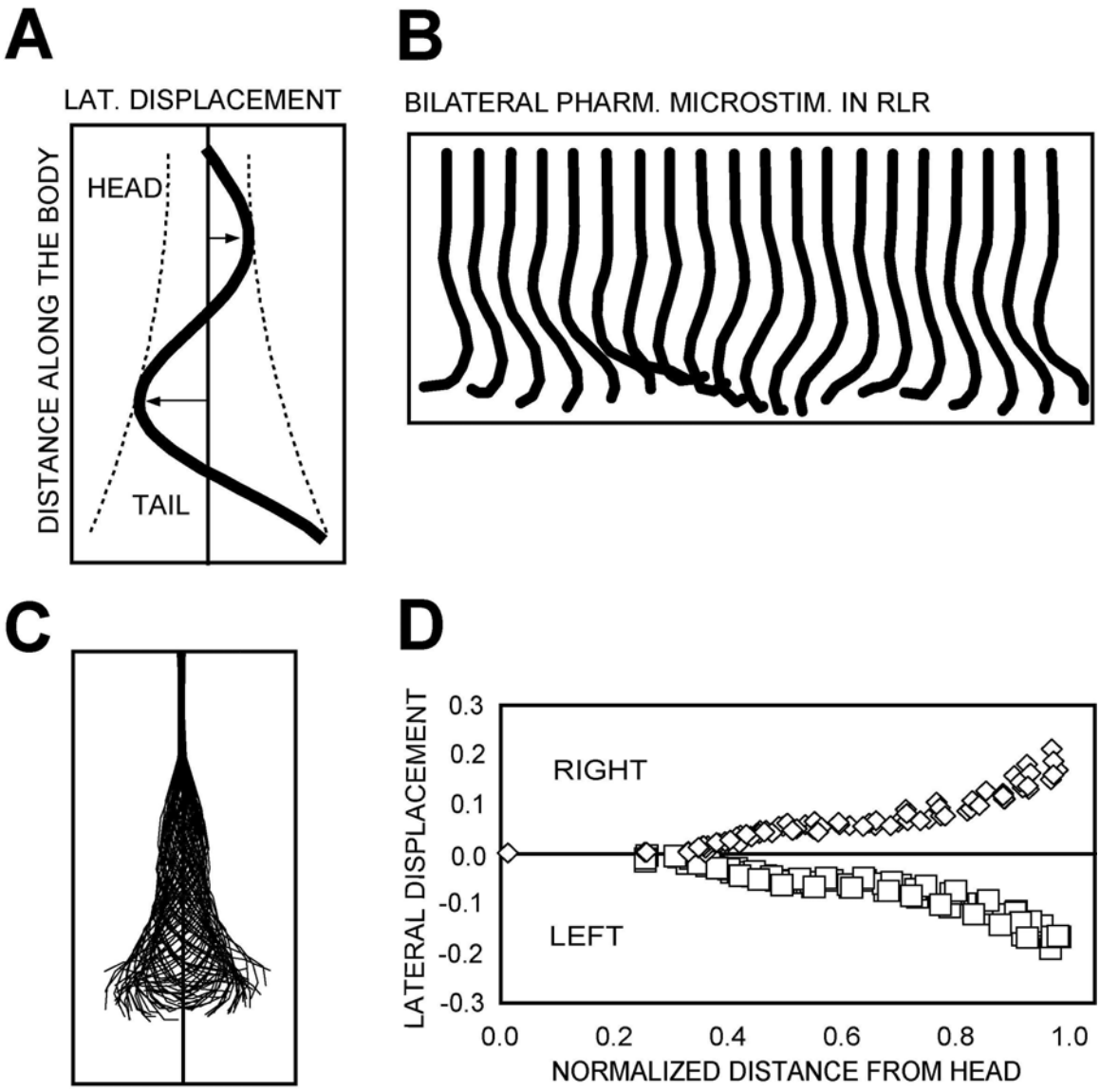
A
LAT. DISPLACEMENT
DISTANCE ALONG THE BODY
HEAD
TAIL
B
BILATERAL PHARM. MICROSTIM. IN RLR
C
D
LATERAL DISPLACEMENT
0.3
0.2
0.1
0.0
-0.1
-0.2
-0.3
RIGHT
LEFT
0.0
0.2
0.4
0.6
0.8
1.0
NORMALIZED DISTANCE FROM HEAD

Figure 3

Figure 4. (A1) Diagram of dorsal view of brain (left) and rostral spinal cord (right) from which HRP had been applied at 20% BL showing traces around cell groups containing HRP-labeled descending brain neurons, symbols representing locomotor "command" areas (DLM, squares; MLR, unfilled circles; RLR, triangles; VMD, filled circles), and the infudibulum (shaded ellipse). Abbreviations: ALV - anterolateral vagal group; ARRN - anterior rhombencephalic reticular nucleus; Di - diencephalon; DLV - dorsolateral vagal group; DLM - dorsolateral mesencephalon; MLR - mesencephalic locomotor region; MRN - mesencephalic reticular nucleus; MRRN - middle rhombencephalic reticular nucleus; PLV - posterolateral vagal group; PON - posterior octavomotor nucleus; PRRN - posterior rhombencephalic reticular nucleus; RLR - rostrolateral rhombencephalon; and VMD - ventromedial diencephalon (modified from Davis and McClellan 1994, Paggett et al., 2004, Sirota et al., 2000). (A2) Outline of brain with traces around the descending trigeminal tract (dV; note that motoneurons in the trigeminal motor nucleus have been omitted for clarity), DLM (squares), MLR (unfilled circles), RLR (triangles), VMD (filled circles), and infundibulum (filled ellipse).

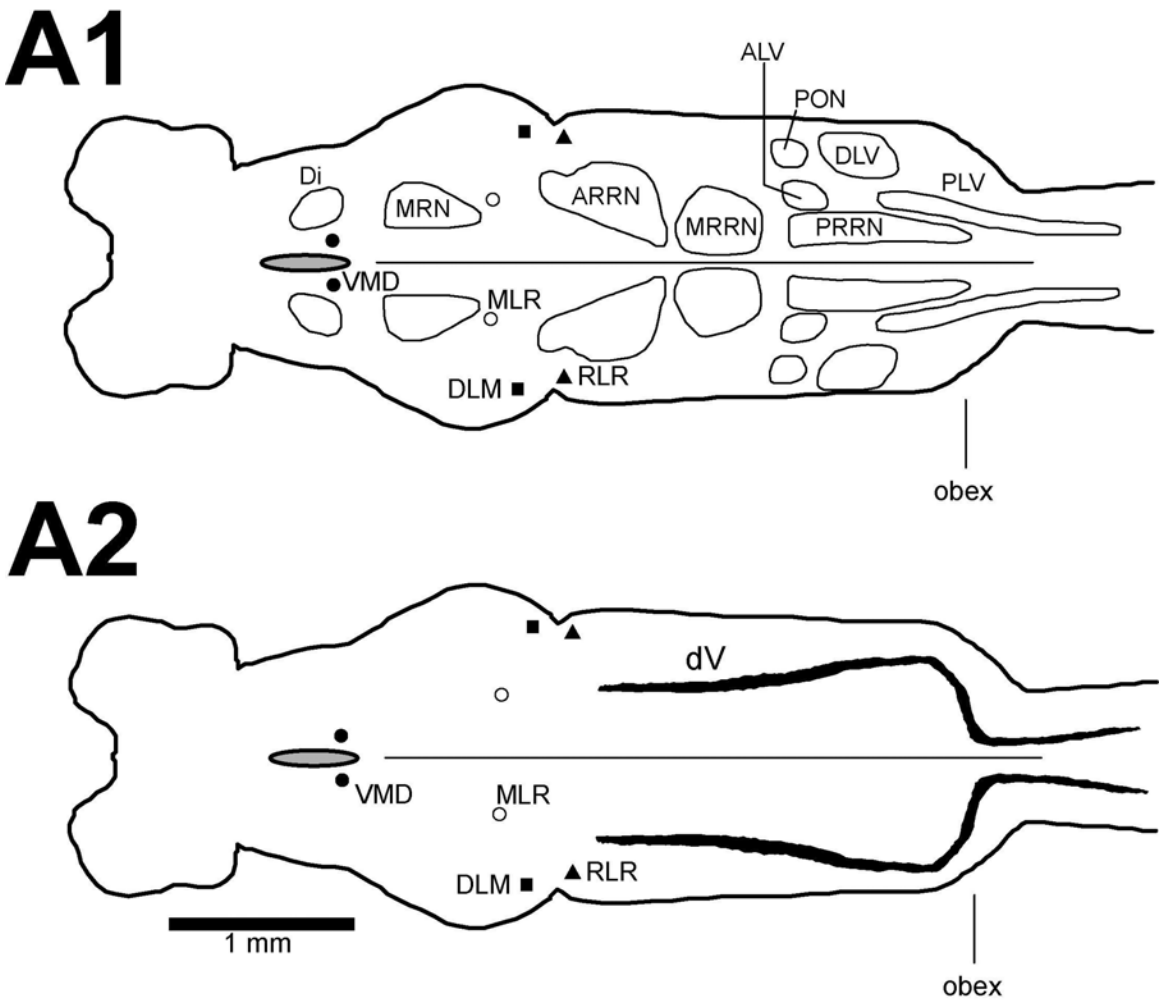
A1
ALV
PON
DLV
PLV
Di
MRN
ARRN
MRRN
PRRN
VMD
MLR
DLM
RLR
obex
A2
dV
VMD
MLR
DLM
RLR
1 mm
obex

Figure 4

Figure 5. (A) Diagram of *in vitro* brain/spinal cord preparation showing bilaterally symmetrical pharmacological microstimulation pipettes (PE_R and PE_L), ventral root recording electrodes (1, 2, and 3), and spinal cord electrode (SC). (B) Schematic of brain with traces around infundibulum (shaded ellipse), reticular nuclei (ARRN, MRN, MRRN, PRRN) and stimulation sites in the VMD (circles), DLM (squares), and RLR (triangles) (see Fig. 4A). (C) Spinal locomotor activity initiated by bilateral microstimulation (PE = pressure ejection pulses) in the (C1) VMD, (C2) DLM, and (C3) RLR consisting of left-right alternation of locomotor burst activity at the same segmental level ($1 \leftrightarrow 2$) and a rostrocaudal phase lag of ipsilateral locomotor burst activity ($2 \rightarrow 3$). During ventral root locomotor activity, substantial neural activity could be recorded at the caudal end of the spinal cord (SC).

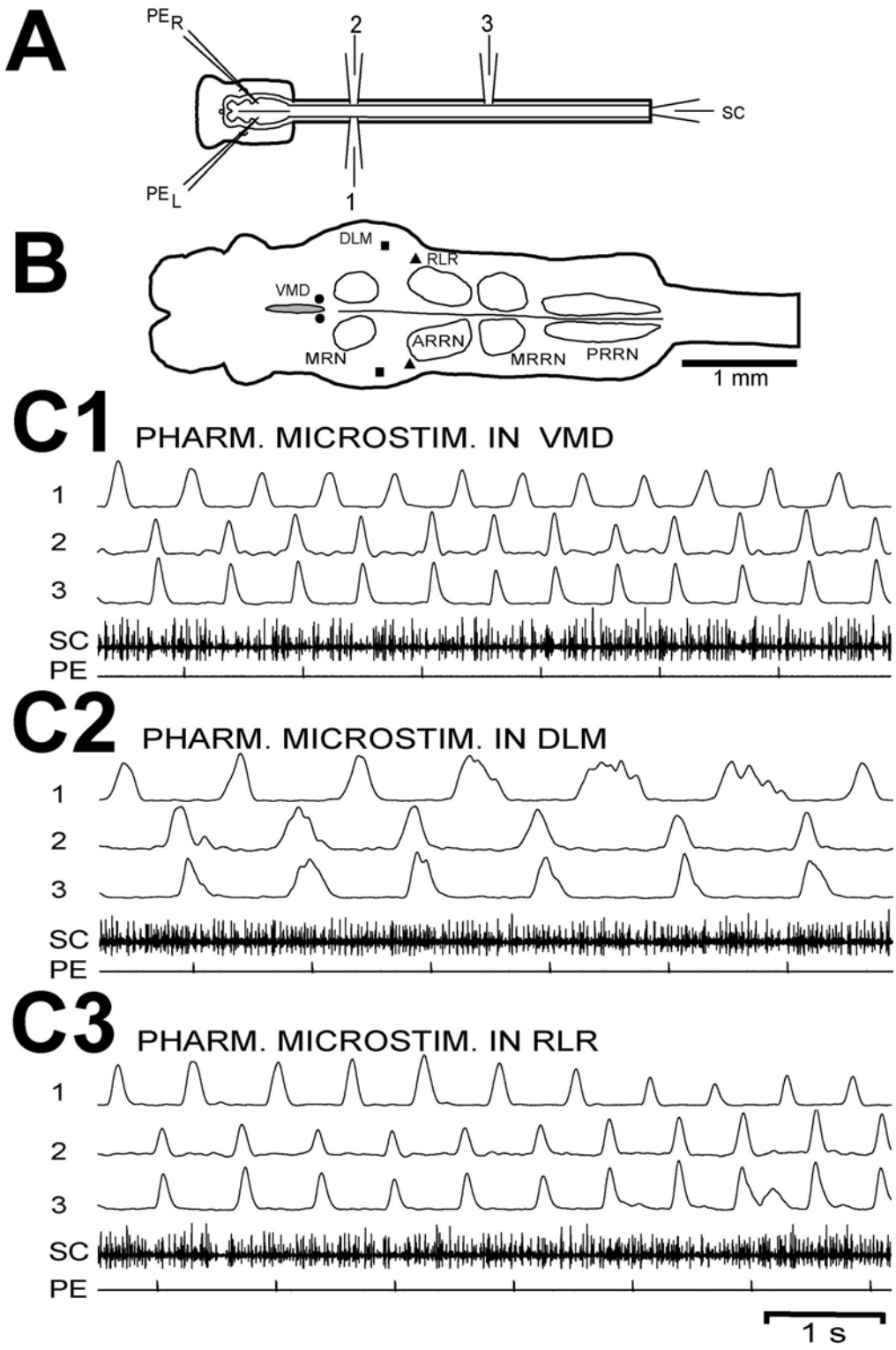
A
PE R
2
3
SC
PE L
1
B
DLM
RLR
VMD
ARRN
MRN
MRRN
PRRN
1 mm
C1
PHARM. MICROSTIM. IN VMD
1
2
3
SC
PE
C2
PHARM. MICROSTIM. IN DLM
1
2
3
SC
PE
C3
PHARM. MICROSTIM. IN RLR
1
2
3
SC
PE
1 s

Figure 5

CHAPTER III

RESULTS

LOCOMOTOR MOVEMENTS INITIATED BY PHARMACOLOGICAL MICROSTIMULATION IN HIGHER LOCOMOTOR COMMAND AREAS

STIMULATION IN HIGHER ORDER LOCOMOTOR AREAS

1. *Pharmacological microstimulation in the RLR.*

In semi-intact preparations from larval lamprey (n = 7), bilateral pharmacological microstimulation in the RLR (Fig. 6A) initiated symmetrical locomotor movements, similar to spontaneous or sensory-evoked episodes of swimming in whole animals. Swimming movements consisted of caudally propagating waves that increased in amplitude with increasing distance from the head (Fig. 6B,D) and that were symmetrical about the midline (Fig. 6C). The envelope of normalized lateral displacement increased with increasing distance from the head (Fig. 6D; Table 1), starting at ~30% BL where the most caudal pins were placed to immobilize the rostral part of the preparation (Fig. 2A). The maximum normalized lateral displacement of the tail was ~0.13 (Table 1), which was not significantly different than the value of 0.12 ± 0.03 produced during free swimming in larval lamprey (ANOVA) (Davis et al., 1993). Stimulation in the RLR in semi-intact preparations initiated swimming movements with an average wavelength of 0.787 and average mechanical phase lag of 0.013 (Table 2; see Methods). These wavelengths and mechanical phase lags were not significantly different than those for swimming in whole animals (n = 6) (ANOVA; Table 2). However, the wavelengths for

swimming in both whole animals and semi-intact preparations (all brain stimulation areas) were significantly less than 1.0 ($P \leq 0.05$, t-test) (Table 2). The above wavelength for RLR-initiated swimming in semi-intact preparations is similar to the value of 0.72 ± 0.07 measured in adult lamprey swimming in a "swim mill" (Williams et al., 1989). It is somewhat remarkable that the kinematics of swimming movements in semi-intact preparations, in which the rostral part of the preparation is immobile, was so similar to those in whole animals. Finally, stimulation in the lateral rhombencephalon, 100-420 μm caudal to and outside the effective stimulation area in the RLR, did not initiate coordinated swimming movements but did produce rhythmic bending, without caudally propagating waves, or C/S-shaped flexures of the body (not shown).

Stimulation in the RLR in semi-intact preparations (n = 15) initiated well-coordinated muscle burst activity consisting of left-right alternation of activity at the same segmental level (1↔2; not shown, see Table 3) and a rostrocaudal phase lag of ipsilateral burst activity (2→3; Fig. 9A). Furthermore, the parameters of RLR-initiated rhythmic muscle burst activity in semi-intact preparations were not significantly different than those during locomotion in normal whole animals (ANOVA; Table 3). It is important to note that in all cases, the mechanical phase lag (Table 2) is greater than the neural phase lag (Table 3). This indicates that the mechanical wave passes down the body at a faster rate than the neural wave (i.e. “slippage”), which must be the case for generation of propulsive force during swimming (Williams, 1986).

2. *Pharmacological microstimulation in the DLM.*

Bilateral stimulation in the DLM (n = 6; Fig. 7A) also initiated symmetrical swimming during which caudally propagating waves increased in amplitude with

increasing distance from the head (Fig. 7B,D) and were symmetrical about the midline (Fig. 7C). The envelope of normalized lateral displacement increased with increasing distance from the head (Fig. 7D, Table 1), and the maximum normalized lateral displacement of the tail was not significantly different than that in freely swimming whole animals. In addition, the wavelengths and mechanical phase lags were not significantly different than those during swimming in whole animals (ANOVA, Table 2). Although the average cycle times of DLM-initiated swimming were significantly longer than those for swimming in normal whole animals (Table 2), the range of cycle times in semi-intact preparations (363-1650 ms) overlap with those in whole animals (130 - 826 ms; Boyd and McClellan, 2002). Stimulation in the rostrolateral mesencephalon, 140-400 μm outside the effective stimulation area in the DLM, elicited uncoordinated movements that did not resemble swimming (not shown).

During swimming movements initiated by stimulation in the DLM, well-coordinated muscle activity (n = 10) consisted of left-right alternation of burst activity (1↔2, Fig. 9C) and a rostrocaudal phase lag (2→3; not shown, see Table 3). The parameters of locomotor activity for DLM-initiated swimming, except cycle time, were not significantly different than those for swimming in whole animals (Table 3). However, the range of cycle times for DLM-initiated swimming activity (375-1446 ms) overlapped with those during swimming in whole animals (Boyd and McClellan, 2002).

3. *Pharmacological microstimulation in the VMD.*

Bilaterally symmetrical stimulation in the VMD (n = 7, Fig. 8A) initiated swimming movements with caudally propagating waves that increased in amplitude toward the tail (Fig. 8B, Fig. 8D, Table 1) and that were symmetrical about the midline (Fig. 8C). The

maximum lateral movements of the tail were not significantly different than those in freely swimming whole animals. The wavelengths and mechanical phase lags for VMD-initiated swimming movements were not significantly different than those during swimming in whole animals (ANOVA, Table 2). Stimulation 120-375 μm lateral to and outside the effective stimulation area in the VMD could elicit movements of the body, but coordinated swimming was never observed (not shown).

Muscle burst activity during VMD-initiated swimming (n = 14) consisted of left-right alternation of activity (1↔2, not shown, Table 3) and a rostrocaudal phase lag (2→3, Fig. 9C, Table 3). The parameters of this locomotor activity were not significantly different than those during swimming in normal whole animals (ANOVA, Table 3).

4. *Unilateral stimulation in higher locomotor areas.*

Unilateral pharmacological stimulation in the RLR (n = 8; Fig. 10A) elicited asymmetrical left-right bending of the body that was skewed away from the side of stimulation in ~70% of the episodes, while in the remaining episodes, swimming movements appeared to be symmetrical. In contrast, unilateral stimulation in the VMD (n = 5; Fig. 10B) or DLM (n = 5; Fig. 10C) could elicit either asymmetrical left-right bending of the body that was skewed toward the side of stimulation or uncoordinated, rhythmic body movements. Since most of these movements did not resemble symmetrical swimming in whole animals, further analyses were not performed.

PHARMACOLOGICAL STIMULATION IN RETICULAR NUCLEI

1. *Locomotor movements initiated by bilaterally symmetrical stimulation.*

Since it was not possible to activate all the RS neurons in a reticular nucleus and simultaneously prevent the spread of the pharmacological agent outside the nucleus,

reticular nuclei were subdivided into smaller areas for the purposes of microstimulation (see Fig. 2B). Bilaterally symmetrical pharmacological microstimulation in certain parts of reticular nuclei (n = 27) initiated symmetrical swimming. For example, stimulation in the anterior ARRN (Fig 11A), posterior MRRN (Fig. 11B), and middle PRRN (Fig. 11C, data for stimulation in the anterior and posterior PRRN not shown) initiated symmetrical swimming movements that were characterized by caudally propagating waves that increased in amplitude towards the tail, as indicated by the envelopes for maximum normalized lateral displacement versus normalized distance from the head (aARRN, Fig. 12A; pMRRN, Fig. 12B; aPRRN, Fig. 12C; mPRRN, Fig. 12D; pPRRN, Fig. 12E; Table 1). The maximum lateral displacements of the tail during swimming movements initiated from reticular nuclei in semi-intact preparations were not significantly different than those in freely swimming whole animals. Kinematic parameters for locomotor movements initiated in semi-intact preparations were not significantly different than those in whole animals, with the exception of cycle times for movements initiated by stimulation in the MRRN and PRRN and mechanical phase lags for movements initiated by stimulation in the MRRN (ANOVA, Table 2). These relatively few differences are not surprising given that only some of the RS neurons in parts of each nucleus probably were activated by focal pressure ejection of excitatory agents (Fig. 2B).

In the present study pharmacological stimulation in the ARRN or PRRN initiated locomotor movements with significantly shorter cycle times than stimulation in the MRRN ($P \leq 0.05$, ANOVA; Table 2). The cycle times of ARRN- or PRRN-initiated swimming movements were not significantly different.

Stimulation in the posterior ARRN and anterior MRRN elicited C-shaped bending or intense flexures of the body, but swimming movements were not observed (not shown). In the posterior ARRN, it is possible that either most of the RS neurons are not involved in the initiation of swimming or insufficient numbers of RS neurons were activated (see Discussion). The anterior MRRN contains several large, identified Müller cells (i.e. "B cells"; Davis and McClellan, 1994b) that project their axons in the medial tracts and are not necessary for the initiation of swimming (McClellan, 1988).

2. *Locomotor muscle activity initiated by bilateral stimulation.*

Microstimulation in the aARRN (Fig. 13A), pMRRN (Fig. 13B), aPRRN (Fig. 14A2), mPRRN (Fig. 14A3), or pPRRN (Fig. 14B2) initiated well-coordinated locomotor activity consisting of left-right alternation of muscle burst activity at the same segmental level (1↔2, Table 3). In general, the parameters of muscle burst activity were not significantly different than those for muscle activity during swimming in whole animals. However, in semi-intact preparations with rostral muscle recording electrodes (1,2; see Fig. 13,14), bilateral stimulation in reticular nuclei often initiated swimming movements and motor activity. In contrast, in preparations with ipsilateral recording electrodes (2,3; see Fig. 2A), stimulation in reticular nuclei usually produced spastic movements or body flexures, possibly because of irritation from the more caudal recording electrode. For this reason, intersegmental phase lags of burst activity were not measured (Table 3). Nonetheless, in the absence of muscle recording electrodes in semi-intact preparations, bilateral stimulation in reticular nuclei reliably initiated well-coordinated swimming movements with wavelengths and mechanical phase lags that were not significantly different than those during actual swimming in whole animals (Table 2, ANOVA).

3. *Unilateral stimulation.*

Unilateral stimulation in reticular nuclei elicited asymmetrical or uncoordinated movements (ARRN, Fig. 15A; MRRN, Fig. 15B; PRRN, Fig. 15C). Further analyses of these movements were not performed since they did not resemble symmetrical swimming in whole animals.

CHAPTER IV

RESULTS

SIZE AND PHARMACOLOGY OF LOCOMOTOR COMMAND AREAS

EXTENT AND PHARMACOLOGY OF HIGHER LOCOMOTOR AREAS

1. *Pharmacological microstimulation in the VMD.*

Bilateral pharmacological microstimulation with 5 mM D-glutamate/D-aspartate in a restricted brain region lateral to the caudal infundibulum, in the ventromedial diencephalon (VMD, Fig. 16A), initiated well-coordinated spinal locomotor activity (n = 5, Fig. 16C1). Brain-initiated ventral root activity consisted of a left-right alternation of burst activity at the same segmental level (1↔2) and a rostrocaudal phase lag of ipsilateral locomotor burst activity (2→3, Fig. 16C1), as previously described (Hagevik and McClellan, 1994).

The VMD locomotor areas were not exactly the same size in different preparations (Fig. 16B, Table 6). In general, the effective brain area tended to be slightly larger in preparations that initiated locomotor activity with the shortest cycle times (data not shown; see Discussion). However, in all preparations (n = 5) the boundary of the VMD locomotor area was clearly demarcated ventrally by the infundibulum and dorsally by areas just outside the locomotor region, where stimulation was ineffective in eliciting spinal locomotor activity (Fig. 16B; see below). In the preparation illustrated, movement of the stimulation pipettes ~50 μM rostral (C3) or lateral (C2) to the most effective stimulation spot (Fig. 16C1) still initiated well-coordinated spinal locomotor activity,

albeit with a slight reduction in the amplitudes of some bursts (Fig. 16C2,16C3). In contrast, stimulation as little as 50 μM outside of the effective stimulation area did not initiate spinal locomotor activity (Fig. 16B, 16C4-16C6), strongly suggesting that pressure ejection focally activates neurons that are relatively close to the tips of the stimulation pipettes.

The parameters of VMD-initiated *in vitro* locomotor activity, for the most part, were not significantly different than those for muscle burst activity during swimming in whole animals (Table 4). Cycle times for VMD-initiated spinal locomotor activity in *in vitro* preparations were significantly longer than those for muscle locomotor burst activity in whole animals (T, Table 4). However, these differences have been noted in previous studies (e.g. McClellan, 1994) and are assumed to be due, in part, to higher excitability of the nervous system in whole animals.

Well-coordinated spinal locomotor activity could be reliably initiated by pharmacological microstimulation in the VMD (n = 3; Fig. 17A) with 5 mM D-glutamate/5 mM D-aspartate (Fig.17B), 0.5 mM NMDA (Fig. 17B2), 1 mM AMPA (Fig. 17B3), or 0.25 mM KA (Fig. 17B4). Thus, all subtypes of glutamate receptors are present and probably participate in VMD-initiated spinal locomotor activity in *in vitro* brain/spinal cord preparations from larval lamprey. Finally, there were no significant differences in the parameters of VMD-spinal locomotor activity initiated by the different agents (Table 5).

2. *Pharmacological microstimulation in the DLM.*

Bilaterally symmetrical pharmacological microstimulation with 5 mM D-glutamate/5 mM D-aspartate within a restricted region in the dorsolateral mesencephalon

(DLM, Fig. 18A) initiated well-coordinated spinal locomotor activity (n = 5, Fig. 18C1). Except for cycle times, the parameters for DLM-initiated locomotor activity were not significantly different than those for swimming activity in whole animals (Table 4). The parameters of DLM- and VMD-initiated locomotor activity, including cycle times, were not statistically different (Table 4).

The boundaries of the DLM were operationally defined caudally by the mesencephalic-rhombencephalic border, and functionally by the dorsal tegmentum, and ventrally by sites outside the DLM that did not elicit locomotor activity (Fig. 18B). However, the DLM locomotor area varied in effective size in different preparations (Fig. 18B, Table 6), similar to that described above for the VMD locomotor area. In all experiments, the rostral and medial extent of the DLM were formed by a clear separation between stimulation sites that initiated spinal locomotor activity (Fig. 18C1-18C3) and relatively nearby stimulation sites surrounding the DLM that were ineffective or elicited uncoordinated ventral root burst activity (Fig 18C4-18C6).

Bilaterally symmetrical pharmacological microstimulation in the DLM (n = 3; squares in Fig. 19A) with 5 mM glutamate/5 mM aspartate (Fig. 19B1), 0.5 mM NMDA (Fig. 19B2), 1 mM AMPA (Fig. 19B3), or 0.25 mM KA (Fig. 19B4) initiated well-coordinated spinal locomotor activity. The parameters of DLM-initiated locomotor burst activity initiated by the different pharmacological agents were not significantly different from each other or from the parameters of burst activity initiated by stimulation with the different agents in the VMD (Table 5).

3. *Pharmacological microstimulation in the RLR.*

Stimulation with 5 mM glutamate/5 mM aspartate in the RLR locomotor area (Fig. 20A) initiated well coordinated spinal locomotor activity consisting of left-right alternation of burst activity at the same segmental level (1↔2) and a rostrocaudal phase lag of ipsilateral locomotor burst activity (2→3) (n = 5, Fig. 20C1). Except for cycle times, the parameters of RLR-initiated ventral root burst activity were not significantly different from those during swimming in whole animals (Table 4). Furthermore, the parameters of spinal locomotor activity initiated by stimulation in the RLR were not significantly different from those elicited by stimulation in the VMD or DLM (Table 4).

In most cases, the size of the RLR tended to be larger in preparations in which *in vitro* locomotor activity had the shortest cycle times (Fig. 20B, Table 6) (data not shown). The boundary of the RLR locomotor area was always clearly demarcated dorsolaterally by the sulcus limitans, rostrally by the mesencephalic-rhombencephalic border, and ventromedially and caudally by stimulation sites just outside the RLR where pharmacological microstimulation was ineffective in initiating spinal locomotor activity (Fig. 20C3-C6).

Pharmacological microstimulation in the RLR locomotor area (triangles, Fig. 21A) with 5 mM D-glutamate/5 mM D-aspartate reliably initiated well-coordinated spinal locomotor activity in all preparations (Fig. 21B1). Microstimulation with 0.5 mM NMDA (Fig. 21B2) and 1 mM AMPA (Fig. 21B3) initiated spinal locomotor in most preparations (4/6 for NMDA and 6/7 for AMPA). In all preparations, repeated stimulation resulted in an increase in the latency in onset of locomotor activity as well as a reduction in the quality of motor activity (data not shown). In contrast, stimulation with

0.25 mM kainate in the RLR locomotor area (Fig. 21B4) was capable of initiating spinal locomotor activity in some preparations (2/6 animals). However, in the remaining preparations (4/6 animals) stimulation resulted in uncoordinated ventral root burst activity that was reduced in amplitude with repeated stimulation episodes (data not shown). There were some differences in the reliability with which the different pharmacological agents could elicit spinal locomotor activity when applied to the above higher locomotor areas. However, the parameters of locomotor burst activity initiated from the RLR, VMD, or DLM by the different pharmacological agents were not significantly different from each other (Table 5).

ORGANIZATION OF HIGHER ORDER LOCOMOTOR AREAS.

First, in *in vitro* brain/spinal cord preparations from larval lamprey (n = 11), bilateral pharmacological microstimulation with 5mM D-glutamate/D-aspartate in the VMD (Fig. 22B1), DLM (Fig. 22C1), or RLR (Fig. 22D1) initiated well-coordinated spinal locomotor activity. Subsequent blockade of synaptic transmission by bath application of a zero calcium Ringer's solution to the brain pool (Pool I, Fig. 22A) abolished VMD (Fig. 22B2), DLM (Fig. 22C2), and RLR (Fig. 22D2) initiated locomotor activity. Furthermore, under these conditions, virtually no activity was recorded at the caudal end of the spinal cord (SC in Fig. 22B2-22D2). These results suggest that these higher order locomotor areas do not directly activate the spinal locomotor networks.

Second, prior to performing a complete transection at the mesencephalic-rhombencephalic border (dark lines, Fig. 23A), pharmacological microstimulation in the RLR locomotor area initiated well-coordinated spinal locomotor activity (n = 3; Fig. 23B1), and mechanical stimulation applied to the oral hood elicited some uncoordinated

burst activity in ventral roots and tonic activity in the caudal end of the spinal cord (Fig. 23B2). Following a complete transection at the mesencephalic-rhombencephalic border (see above), bilateral pharmacological microstimulation in the same RLR locomotor area elicited very little ventral root activity and tonic activity at the caudal end of the spinal cord (Fig. 23C1). Mechanical stimulation applied to the oral hood still elicited some brief ventral root burst and caudal spinal cord activity (Fig. 23C2).

CHAPTER V

RESULTS

DISRUPTION OF RECIPROCAL CONNECTIONS IN THE SPINAL CORD

DISRUPTION RECIPROCAL COUPLING BETWEEN LOCOMOTOR NETWORKS IN THE CAUDAL CORD

1. *Movements and muscle activity in whole animals.*

Whole animals with longitudinal midline lesions in the caudal spinal cord (30-50% BL; horizontal line in Fig. 24A; n = 6) but without spinal cord transection at 50% BL were able to swim and exhibit forward progression. However, several very obvious deficits were noted based on behavioral observations: 1) smaller than normal rhythmic lateral tail displacements, partly due to a slight bend (i.e. constant flexure) in the lesioned region of the body to one side, in the ventral direction, or a combination of both; 2) lower than normal velocity of swimming; and 3) difficulty with directional control of swimming, with a tendency to roll laterally either to the right or left. Also, most of the lesioned animals appeared to have difficulty burrowing, and all lesioned animals were generally inactive unless stimulated. In a separate group of whole animals with both caudal midline spinal lesions as well as spinal cord transections at 50% BL (vertical line in Fig. 24A, T; n = 6), the behavioral deficits were similar but somewhat more pronounced compared to those in animals with only midline lesions. In particular, lateral

displacement of the tail during swimming was reduced, forward progression was slower than normal, and stimulation of the tail no longer elicited episodes of swimming.

Despite obvious deficits in swimming movements, well-coordinated locomotor muscle activity was observed in whole animals with midline lesions of the caudal spinal cord alone (n = 6) in response to tactile or electrical stimulation of the oral hood or tail (Fig. 24B). Locomotor activity consisted of left-right alternation of muscle burst activity in the rostral body (Fig. 24B, 1↔2) that usually was accompanied by caudal alternating burst activity (3↔4; see following text), which resulted in a rostrocaudal phase lag of ipsilateral muscle burst activity (1→4 and 2→3). Furthermore, the parameters of this rhythmic muscle activity were not significantly different than those during swimming in normal control animals (Table 7; ANOVA).

In a separate group of whole animals with both midline lesions in the caudal spinal cord as well as spinal cord transections at 50% BL (vertical line in Fig. 24A, T; n = 6), locomotor muscle activity was present in the rostral and usually the caudal body (Fig. 24C1), and the parameters of this activity were not significantly different than those during swimming in normal control animals (Table 7). These results indicate that ascending inputs originating caudal to the midline spinal cord lesions were not necessary either for the generation or phasing of muscle activity from locomotor networks in caudal hemi-spinal cords.

Although left-right alternating muscle burst activity in the caudal body was observed in all preparations, without (n = 6) or with (n = 6) a spinal cord transection at 50% BL, this burst activity often became smaller in amplitude, or was absent, as swimming frequency and speed decreased. In addition, in all preparations, without or

with transections, some of the caudal motor activity consisted of short "burstlets" at ~20-40 Hz and that occurred either together in "packets" to form longer bursts (Fig. 24C2, *), continuously during rostral burst activity (Fig. 24C3, *), or alone in the absence of rostral activity (not shown), usually with relatively small amplitudes. The prevalence of this "burstlet" activity was variable not only between animals but also for a given animal. Finally, right and left "burstlet" activity did not appear to show a phase preference. Because of the very high frequency of this activity, it was not analyzed further.

In animals with "short" midline lesions in the caudal spinal cord (35–45% BL; data not shown), locomotor muscle activity occurred in rostral and usually caudal regions of the body, either with (n = 6) or without (n = 6) spinal cord transections at the caudal extent of the midline lesions (45% BL) (data summarized in Table 7). Since muscle activity patterns in animals with "short" (35-45% BL) and "long" (30-50% BL) midline lesions in the caudal spinal cord were similar, results from animals with "long" midline lesions are emphasized here.

2. *Motor activity in in vitro preparations.*

In vitro brain/spinal cord preparations (Fig. 25A) were used to determine whether burst activity could be generated in caudal hemi-spinal cords in the absence of mechanosensory inputs. Prior to spinal cord lesions, pharmacological microstimulation in brain locomotor areas (PE; see Methods) initiated well-coordinated control locomotor activity consisting of left-right alternating burst activity in rostral and caudal ventral roots ($1\leftrightarrow2$, $3\leftrightarrow4$) that resulted in a rostrocaudal phase lag (Fig. 25B1, $1\rightarrow4$, $2\rightarrow3$; n = 13). Following a midline lesion of the caudal spinal cord (30-50% BL; horizontal line in Fig. 25A; n = 13), left-right alternating burst activity was initiated in the intact, rostral ($1\leftrightarrow2$)

spinal cord and usually was accompanied by similar activity in the lesioned, caudal (3↔4) cord (Fig. 25B2). In 10 of 13 preparations following midline spinal cord lesions, there was a clear reduction in the amplitudes of caudal ventral root bursts, but since the ventral root electrodes were removed to make the lesions, changes in recording conditions cannot be excluded as a cause for this difference. In addition, caudal burst activity often was reduced substantially in amplitude or absent during rhythmic activity with relatively long cycle times. Following a spinal transection at 50% BL, at the caudal extent of the midline lesion, to eliminate ascending inputs from more caudal spinal neural networks, alternating burst activity could be initiated in the rostral and caudal spinal cord (Fig. 25B3; n = 7), similar to that prior to the transection. Under the conditions in Figure 25B2 and 25B3, most of the parameters of ventral root burst activity were not significantly different than those for control locomotor activity prior to performing spinal lesions. However, intersegmental phase lags (ϕ_{INT}) and burst proportions for caudal activity (BP_{caud}) were significantly smaller and significantly larger, respectively, than those for control locomotor activity (Table 8; ANOVA). In addition, the envelopes of integrated burst activity in the midline lesioned caudal cord usually were more erratic than those for rostral burst activity. Finally, when the *in vitro* motor activity following midline lesions was integrated with a relatively short time constant (~12.5 ms), short "burstlets" similar to those present in the EMG activity in whole animals (Fig. 24D) were not observed. Thus, these "burstlets" in the EMG activity may have been due, in part, to sensory feedback or differences in the excitability of whole animal and *in vitro* preparations.

Several issues were addressed regarding locomotor activity in *in vitro* preparations with longitudinal spinal cord lesions. First, is *in vitro* burst activity in caudal hemi-spinal cords (Fig. 25B2 and 25B3, 3↔4) generated by spinal CPGs and does it represent swimming behavior? During normal swimming in the lamprey, rostrocaudal phase lags are relatively constant versus cycle times (Wallén and Williams, 1984; Boyd and McClellan, 2002). In the present study, analysis of motor activity similar to that in Figure 25B2 and 25B3 indicated that rostrocaudal phase lags did not change significantly versus cycle times either without (n = 13) or with (n = 7) spinal cord transections at 50% BL ($p > 0.12$; regression analysis). However, these phase lags were significantly less than those for control locomotor activity ($p \leq 0.05$; ANOVA) and were not significantly different than zero ($p > 0.05$; t-Test). Thus, these data suggest that rhythmic burst activity following a midline lesion in the caudal spinal cord would not give rise to well-coordinated locomotor movements.

Second, can isolated caudal hemi-spinal cords alone generate burst activity (Fig. 25B2 and 25B3, 3↔4) in response to descending inputs from the brain? It should be noted that in unlesioned *in vitro* brain/spinal cord preparations from larval lamprey in which synaptic transmission is blocked in the rostral spinal cord, stimulation in brain locomotor areas results in direct activation of locomotor networks in the caudal spinal cord and initiation of alternating locomotor burst activity (McClellan, 1994). In the present study, in partitioned *in vitro* brain/spinal cord preparations with longitudinal midline lesion in the caudal spinal cord (30%-50% BL, horizontal line, Fig. 26A), alternating burst activity could be initiated in the intact, rostral (1↔2) and lesioned, caudal (3↔4) regions of the spinal cord (Fig. 26B1), as described above. When chemical

synaptic transmission was blocked in rostral, intact regions of the spinal cord with a low-calcium Ringer's solution (Pool II; see Methods), rhythmic locomotor-like burst activity could no longer be initiated in the caudal hemi-spinal cords, which were bathed in normal Ringer's solution (Fig. 26B2, 3 and 4; n = 6). The relatively small, unpatterned upward deflections in the recordings from caudal ventral roots (3,4; Fig. 26B2) do not appear to be locomotor bursts because similar but lower amplitude upward deflections were also present in recordings from the rostral spinal cord that was bathed in low calcium Ringer's solution (1 and 2; data not shown). Since some unpatterned activity was present in caudal ventral roots under these conditions, it is unlikely that CPG interneurons were rhythmically active but subthreshold for activating and/or modulating motoneurons. Returning normal Ringer's solution to the rostral spinal cord pool restored alternating burst activity in both rostral and caudal regions of the cord (Fig. 26B3). These results suggest that caudal hemi-spinal cords cannot generate rhythmic burst activity in response to descending activation from the brain alone but also require descending propriospinal inputs from intact regions of the rostral spinal cord.

Third, are descending propriospinal inputs alone from intact, rostral spinal locomotor networks sufficient to activate rhythmic burst activity in locomotor networks in left and right caudal hemi-spinal cords? In *in vitro* preparations with midline lesions of the caudal spinal cord (30-50% BL) and spinal cord transections at 50% BL, the very rostral spinal cord was transected at the brain-spinal cord border. Application of 1.0 mM D-glutamate to the entire spinal cord (see McClellan, 1990b) elicited some rhythmic burst activity in the rostral spinal cord (1↔2) but only tonic activity in the caudal hemi-spinal cords (Fig. 26B4; n = 5). Typically, pharmacologically activated swimming

rhythms in the isolated spinal cords of larval lamprey are more erratic and have lower signal-to-noise ratios than those from adult lamprey (McClellan, 1990b; Cohen et al., 1990). Taken together, the above results suggest that functionally isolated left and right caudal hemi-spinal cords cannot generate rhythmic locomotor burst activity in larval lamprey. Both descending activation from the brain and descending propriospinal inputs from intact, rostral regions of the cord appear to be necessary for generation of rhythmic burst activity in caudal hemi-spinal cords.

DISRUPTION OF RECIPROCAL COUPLING IN THE ROSTRAL CORD

1. *Movements and muscle activity in whole animals.*

In whole animals with longitudinal midline lesions in the rostral spinal cord (8-30% BL, horizontal line in Fig. 27A; n = 10) but without spinal cord transections at 30% BL, swimming-like movements resulted in forward progression of the body. However, there were several clear deficits based on behavioral observations: 1) lower than normal velocity of swimming, 2) difficulty with directional control of swimming (e.g. turning); 3) a tendency to roll laterally either to the right or left, partly due to a slight bend (i.e. constant flexure) in the lesioned region of the body to one side, in the ventral direction, or a combination of both; and 4) relatively short episodes of sensory-evoked swimming. Although none of the lesioned animals burrowed in the sand at the bottom of their aquaria, they could be spontaneously active, and swimming behavior was often observed. In contrast, whole animals with both rostral midline spinal lesions (8-30% BL) and spinal cord transections at 30% BL (vertical line in Fig. 27A, T; n = 6) did not produce locomotor movements. In these animals, stimulation of the oral hood elicited tonic

flexure responses above the transection, but rhythmic left-right bending of the rostral part of the body was never observed. Similarly, stimulation of the tail elicited flexure activity below the transection, but did not initiate swimming movements.

Whole animals with rostral midline spinal lesions but without spinal cord transections at 30% BL generated two possible patterns of muscle burst activity (n = 10 animals). In 6 of 10 animals, tactile or electrical stimulation of the oral hood could initiate left-right alternating muscle burst activity in caudal (3↔4) and usually rostral (1↔2; see following text) regions of the body, and relatively small rostrocaudal phase lags for ipsilateral burst activity (Fig. 27B, 1-4, 2-3). During relatively slow undulatory movements, rostral muscle burst activity often was reduced substantially in amplitude or was absent. Most of the parameters of rhythmic burst activity were not significantly different than those during swimming in normal control animals (Table 7). However, intersegmental phase lags (ϕ_{INT}) were significantly less than those for control locomotor activity (Table 7; $p \leq 0.05$; ANOVA) and were not significantly different than zero ($p > 0.05$; t-test). In 4 of 10 animals, alternating muscle burst activity usually was present only in the caudal regions of the body in which the spinal cord was intact (Table 7). In the rostral body, where left-right coupling was interrupted by a midline spinal lesion, muscle activity was always present during swimming-like movements but generally was tonic and not correlated with activity in the caudal regions of the body (data not shown).

In a separate group of whole animals with both midline lesions in the rostral spinal cord (8-30% BL) and spinal cord transections at 30% BL (vertical line in Fig. 27A, T, n = 6), coordinated muscle burst activity usually did not occur (Fig. 27C). Specifically, stimulation of the oral hood (Fig. 27C, triangle) evoked tonic flexure muscle

activity (however, see following text) that often resulted in movements of the head away from the stimulus, but undulatory or locomotor-like movements never occurred. Since muscles in the rostral body could display some unpatterned activity under these conditions, it is unlikely that CPG interneurons were rhythmically active but subthreshold for activating and/or modulating motoneurons. Tactile or electrical stimulation below the spinal cord transection site at 30% BL elicited only tonic flexure muscle activity in the tail, but coordinated locomotor muscle activity was never observed (data not shown).

It is important to demonstrate that the absence of locomotor muscle activity in whole animals with both rostral midline spinal lesions and spinal cord transections (Fig. 27C) was not due to damage of motor networks in the rostral spinal cord. As an additional test, following midline lesions in the rostral spinal cord (8–30 % BL; horizontal line in Fig. 28A), muscle recordings were made in the same animals before and after spinal cord transections were performed at 30% BL (T, vertical line in Fig. 28A; n = 3). Following midline lesions of the rostral spinal cord alone, left-right alternating muscle burst activity could occur in both the rostral and caudal regions of the body (Fig. 28B1), as described above. Subsequently, the same animals were re-anesthetized, and spinal cord transections were performed at 30% BL. Following recovery from anesthesia, stimulation of the oral hood usually elicited only tonic flexure muscle activity in the rostral part of the body (Fig. 28B2, 1 and 2; however, see following text), but undulatory or locomotor-like movements were never observed. These results suggest that following midline lesions of the rostral spinal cord, the absence of alternating muscle burst activity in these parts of the body (Figs. 27C and 28B2) was not due to damage of spinal motor networks (see Discussion).

In all preparations with rostral midline lesions of the spinal cord, with or without spinal cord transections at 30% BL, some of the rostral motor activity consisted of short "burstlets" at ~15-30 Hz. Again, the prevalence of "bursetlet" activity varied within an animal and between animals. In addition, in 2 of 9 preparations following spinal cord transection at 30% BL, occasionally some left-right alternating activity occurred in rostral musculature that consisted of groups of these relatively short "burstlets" (Fig. 28B3i and 28B3ii).

2. *Motor activity in in vitro preparations.*

In vitro brain/spinal cord preparations were used to determine whether burst activity could be generated in rostral hemi-spinal cords in the absence of mechanosensory inputs. Prior to performing lesions, pharmacological microstimulation in brain locomotor areas (see Methods) initiated well-coordinated control spinal locomotor activity (Figs. 29B1, 30B1, and 31B1). Following midline lesions in the rostral spinal cord (8-30% BL; horizontal line in Figs. 39A and 30A), stimulation in brain locomotor areas usually elicited weak tonic activity in rostral ventral roots (1 and 2) and left-right alternating burst activity in caudal ventral roots (Figs. 29B2 and 30B2, 3↔4; n = 6). In 5 of 6 preparations following midline lesions, there was a clear reduction in the amplitudes of caudal burst activity, but since the ventral root electrodes were removed to make the lesions, a change in recording conditions cannot be excluded. No short "burstlets", similar to those in the EMG activity (Fig. 28B3), were observed when the *in vitro* motor activity was integrated with a relatively short time constant (~12.5 ms). Thus, these "burstlets" in the EMG activity may have been due, in part, to sensory feedback or differences in the excitability of whole animal and *in vitro* preparations. The parameters

of the alternating burst activity recorded from the caudal, intact spinal cord were not significantly different than those during locomotor activity recorded prior to performing midline lesions (Table 8). Occasionally, a few upward deflections in the rostral ventral root activity appeared to be 1:1 with caudal burst activity, but these occurrences were too infrequent to analyze and were considered atypical. The relatively small, unpatterned upward deflections in the rostral ventral root recordings (Fig. 29B2, 1 and 2) probably are not locomotor bursts because similar activity can be recorded during stimulation in brain locomotor areas from ventral roots in a region of spinal cord that is bathed in low calcium Ringer's solution (e.g. McClellan, 1994).

For *in vitro* preparations with both rostral midline spinal lesions and spinal cord transections at the caudal extent of these lesions (T; vertical line in Figs. 29A, 30A, and 31A), two possible types of motor activity could be produced (n = 10). In 3 of 10 preparations, pharmacological microstimulation in brain locomotor areas elicited only tonic or uncoordinated activity in rostral ventral roots (Fig. 29B3, 1 and 2). In 7 of 10 preparations, brain stimulation elicited slow rhythmic ventral root activity, consisting of left-right alternation (Fig. 30B3 and 31B2, 1↔2) and nearly synchronous ipsilateral burst activity (Fig. 31B2, 2 and 3). In theory, the rhythmicity of this slow burst activity might have resulted from three possible mechanisms: 1) generated by spinal locomotor networks; 2) generated as a result of ascending-descending feedback loops between the spinal cord and brain; or 3) generated by neural circuits in the brain. First, the slow burst activity had average cycle times of almost 4 s that were significantly longer than those for control *in vitro* locomotor activity ($p = 0.01$; unpaired t-test with Welch correction). Furthermore, the burst proportions of this activity were significantly larger than those for

pre-lesion control locomotor activity ($p \leq 0.05$; Table 8; ANOVA), and intersegmental phase lags (Fig. 31B2, 2→3) were significantly less than those for control *in vitro* locomotor activity ($p \leq 0.001$; ANOVA). These data suggest that the slow burst activity probably would not give rise to well-coordinated locomotor movements. Second, under conditions in which chemical microstimulation in brain locomotor areas elicited slow rhythmic ventral root burst activity in rostral hemi-spinal cords (Fig. 31B2), alternating burst activity also was present in right and left fascicles at the caudal end of the spinal cord (Fig. 32A and 32B1). When a low calcium Ringer's solution was applied to the spinal cord (Pool II), stimulation in the same brain areas still elicited alternating burst activity in spinal fascicles (Fig. 32B2; $n = 4$). These results suggest that under the present experimental conditions, neural circuits in the brain contributed substantially to the rhythmicity of the slow bursting pattern that was observed in left and right rostral hemi-spinal cords (Figs. 30B3 and 31B2).

Figure 6. (A) Diagram of brain showing bilaterally symmetrical microstimulation sites in the RLR (filled squares). (B) Sequential swimming movements, left to right, in a semi-intact preparation initiated by bilateral pharmacological microstimulation in the RLR (same animal as A; T = 990 ms, IFI = 100 ms) (see legend for Fig. 3). (C) Sequential, superimposed body movements and (D) plot of normalized lateral displacement versus normalized distance along the body from same episode as B (open circles are means and short vertical lines are SDs) (see Fig. 3).

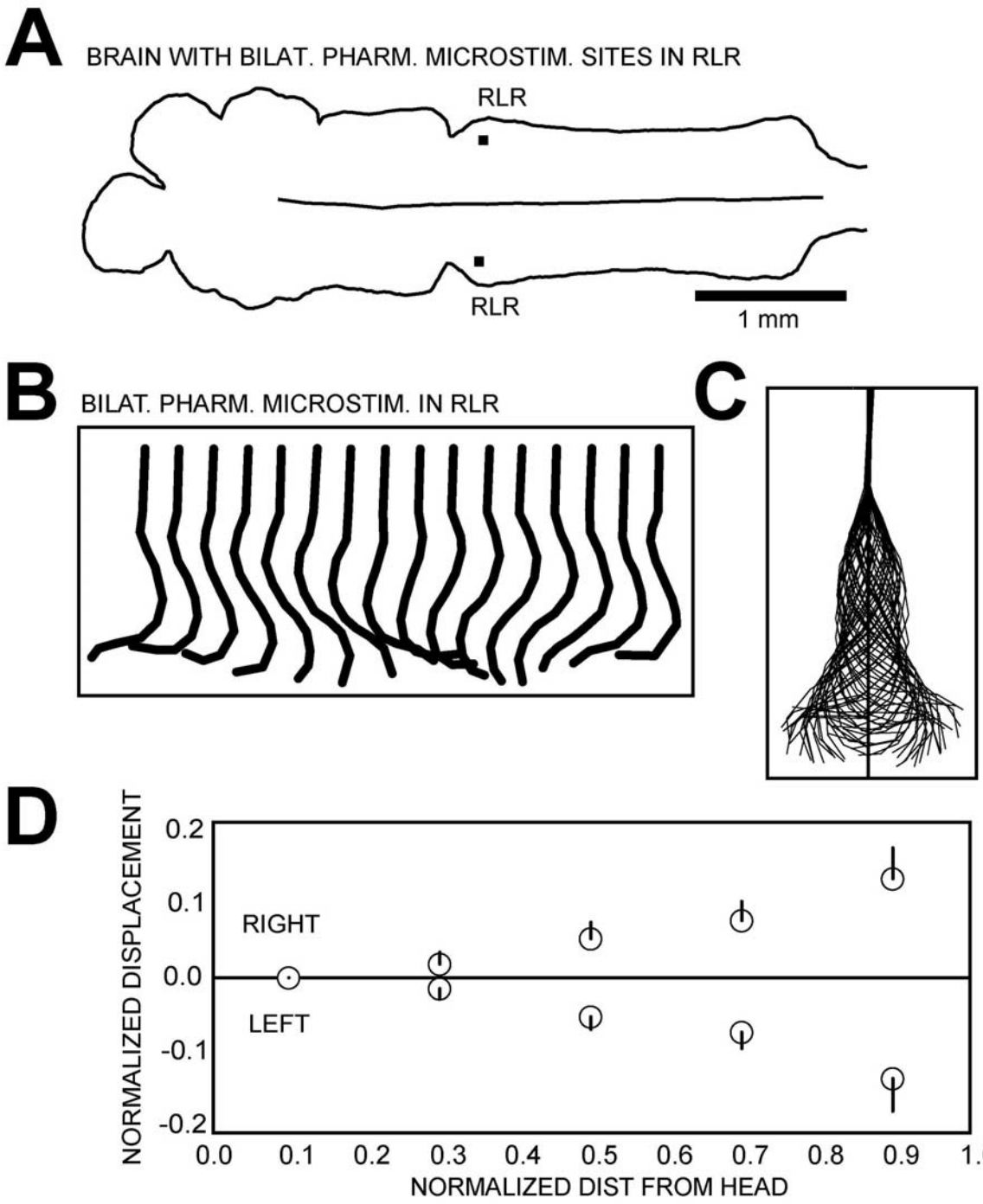
A
BRAIN WITH BILAT. PHARM. MICROSTIM. SITES IN RLR
RLR
RLR
1 mm
B
BILAT. PHARM. MICROSTIM. IN RLR
C
D
NORMALIZED DISPLACEMENT
0.2
0.1
0.0
-0.1
-0.2
RIGHT
LEFT
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1.0
NORMALIZED DIST FROM HEAD

Figure 6

Figure 7. (A) Brain diagram with microstimulation sites in the DLM (filled squares). (B) Plot of sequential, movements, left to right, initiated by bilateral pharmacological microstimulation in the DLM (same animal as A; T = 429 ms, IFI = 33 ms). (C) Sequential, superimposed body movements and (D) lateral displacement versus distance along the body from same episode as B (see Figs. 3,6).

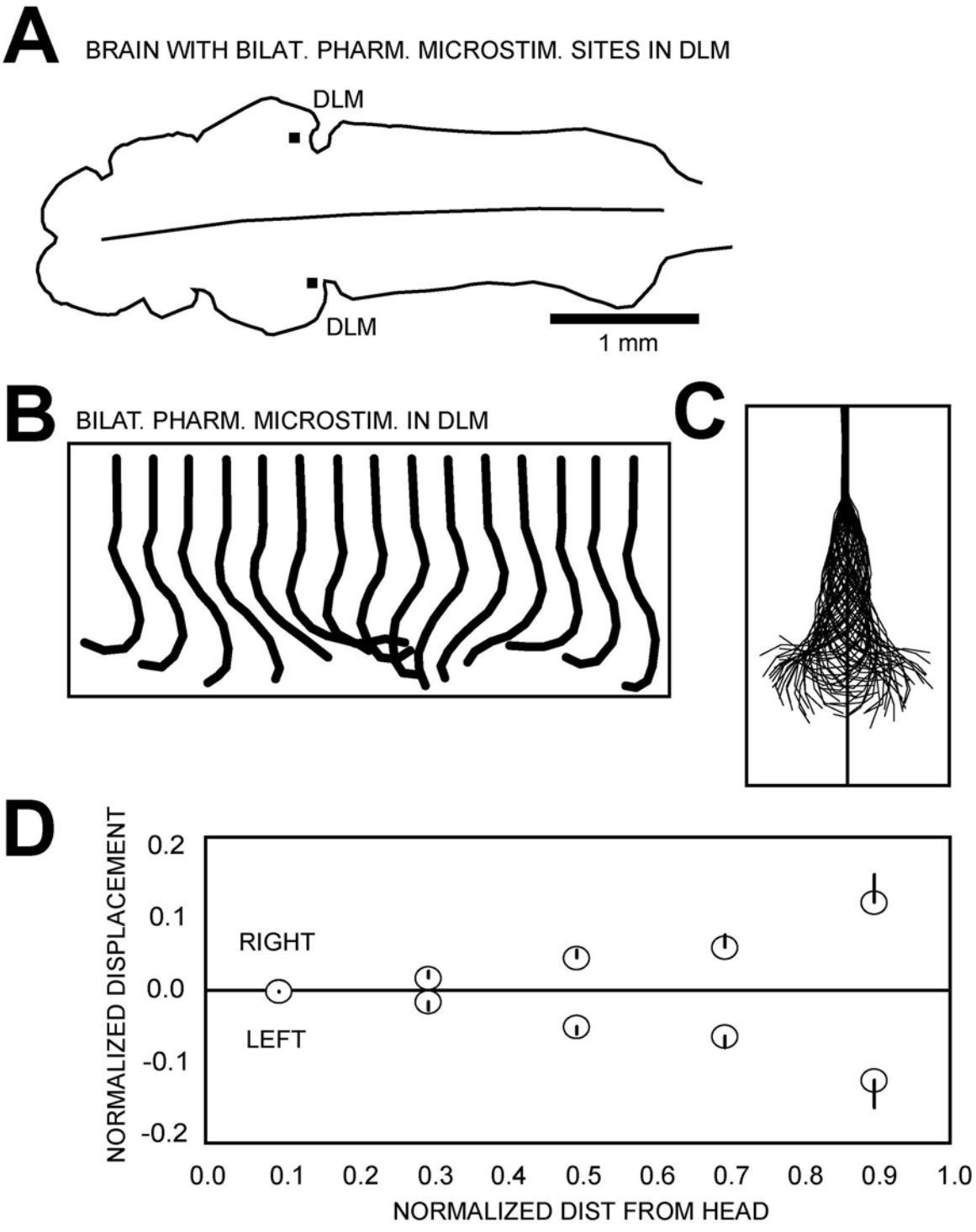
A
BRAIN WITH BILAT. PHARM. MICROSTIM. SITES IN DLM
DLM
DLM
1 mm
B
BILAT. PHARM. MICROSTIM. IN DLM
C
D
NORMALIZED DISPLACEMENT
0.2
0.1
0.0
-0.1
-0.2
RIGHT
LEFT
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1.0
NORMALIZED DIST FROM HEAD

Figure 7

Figure 8. (A) Diagram of brain showing microstimulation sites in the VMD (filled squares). (B) Sequential movements, left to right, initiated by bilateral pharmacological microstimulation in the VMD (same animal as A; T = 396, IFI = 33 ms). (C) Sequential, superimposed body movements and (D) lateral displacement versus distance along the body from same episode as B (see Figs. 3,6).

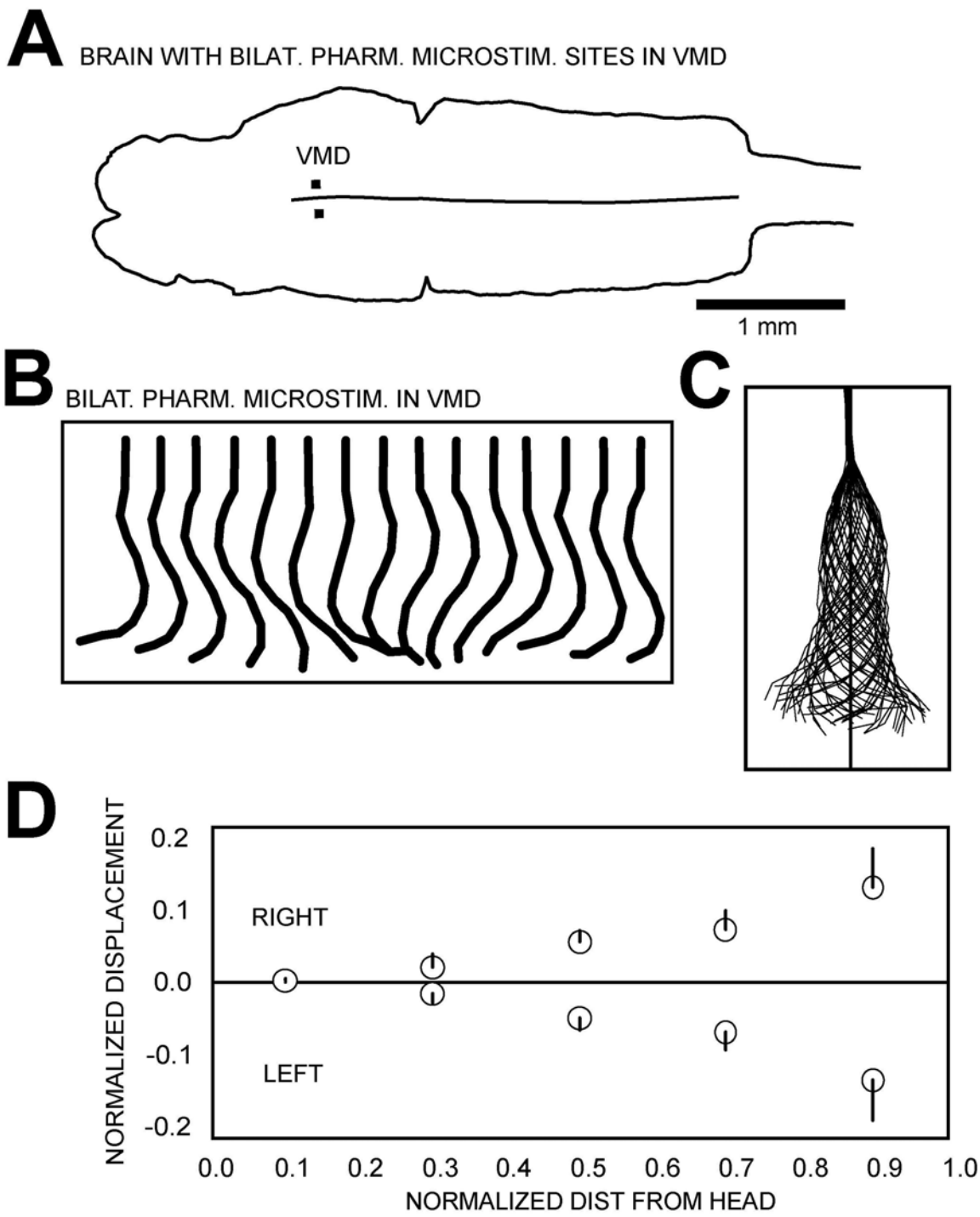
A
BRAIN WITH BILAT. PHARM. MICROSTIM. SITES IN VMD
VMD
1 mm
B
BILAT. PHARM. MICROSTIM. IN VMD
C
D
NORMALIZED DISPLACEMENT
0.2
0.1
0.0
-0.1
-0.2
RIGHT
LEFT
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1.0
NORMALIZED DIST FROM HEAD

Figure 8

Figure 9. (A) Bilateral pharmacological microstimulation in the RLR initiated swimming movements and muscle activity, which consisted of a rostrocaudal phase lag (2→3) of burst activity on the same side (2 at 49%BL, and 3 at 62% BL; see Fig. 2A). (B) Stimulation in the VMD initiated swimming muscle activity consisting of left-right alternation (1↔2) of burst activity at the same segmental level (1,2 at 49% BL; see Fig. 2A). (C) Stimulation in the DLM initiated swimming muscle activity with a rostrocaudal phase lag (2→3; 2 at 49% BL, and 3 at 62% BL).

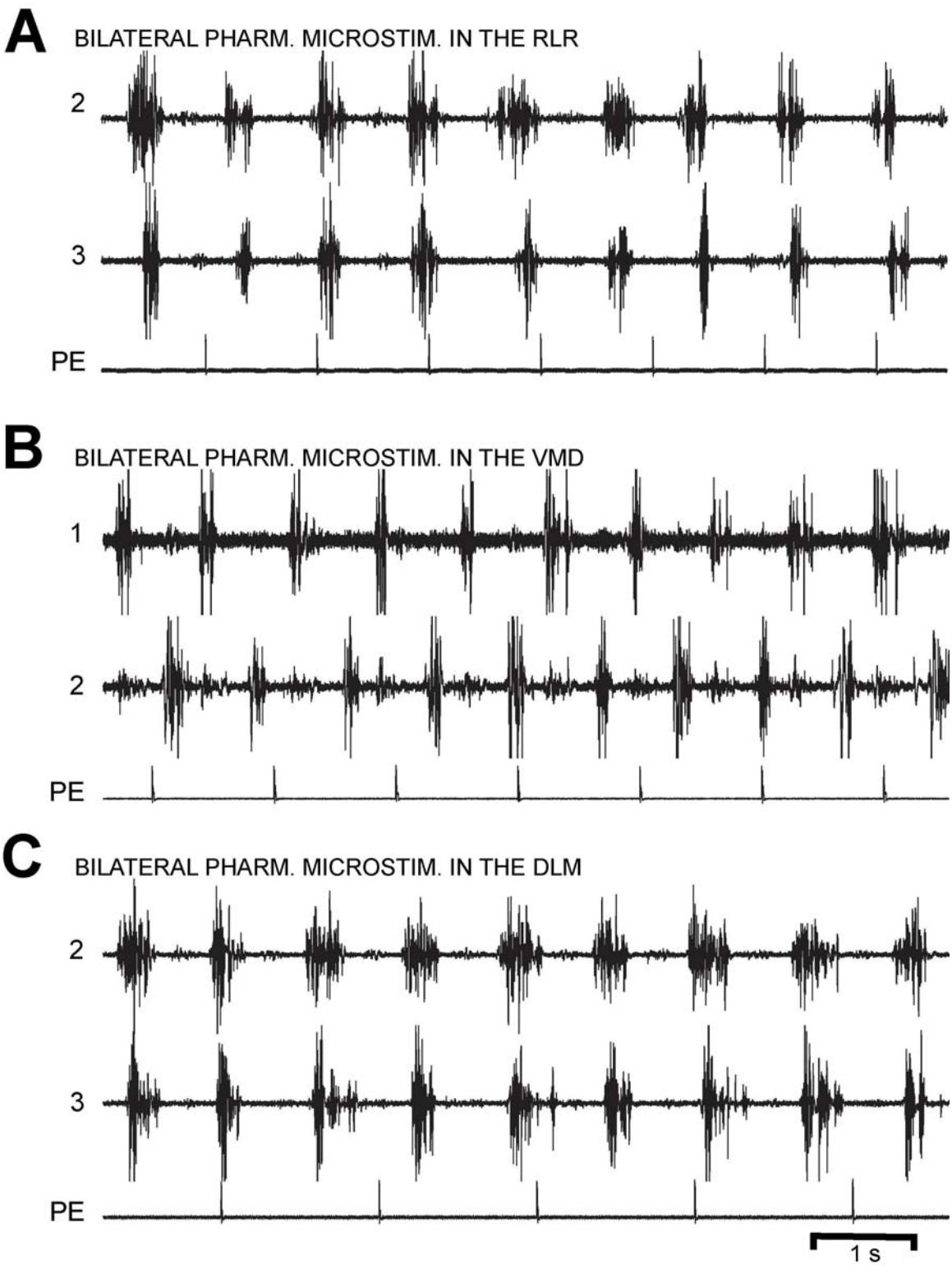
A
BILATERAL PHARM. MICROSTIM. IN THE RLR
2
3
PE
B
BILATERAL PHARM. MICROSTIM. IN THE VMD
1
2
PE
C
BILATERAL PHARM. MICROSTIM. IN THE DLM
2
3
PE
1 s

Figure 9

Figure 10. Sequential asymmetrical movements, left to right, elicited by unilateral pharmacological microstimulation in the (A) left RLR (T = 660 ms, IFI = 67 ms), (B) right VMD (T = 528 ms, IFI= 67 ms), and (C) right DLM (T = 660 ms, IFI = 67 ms).

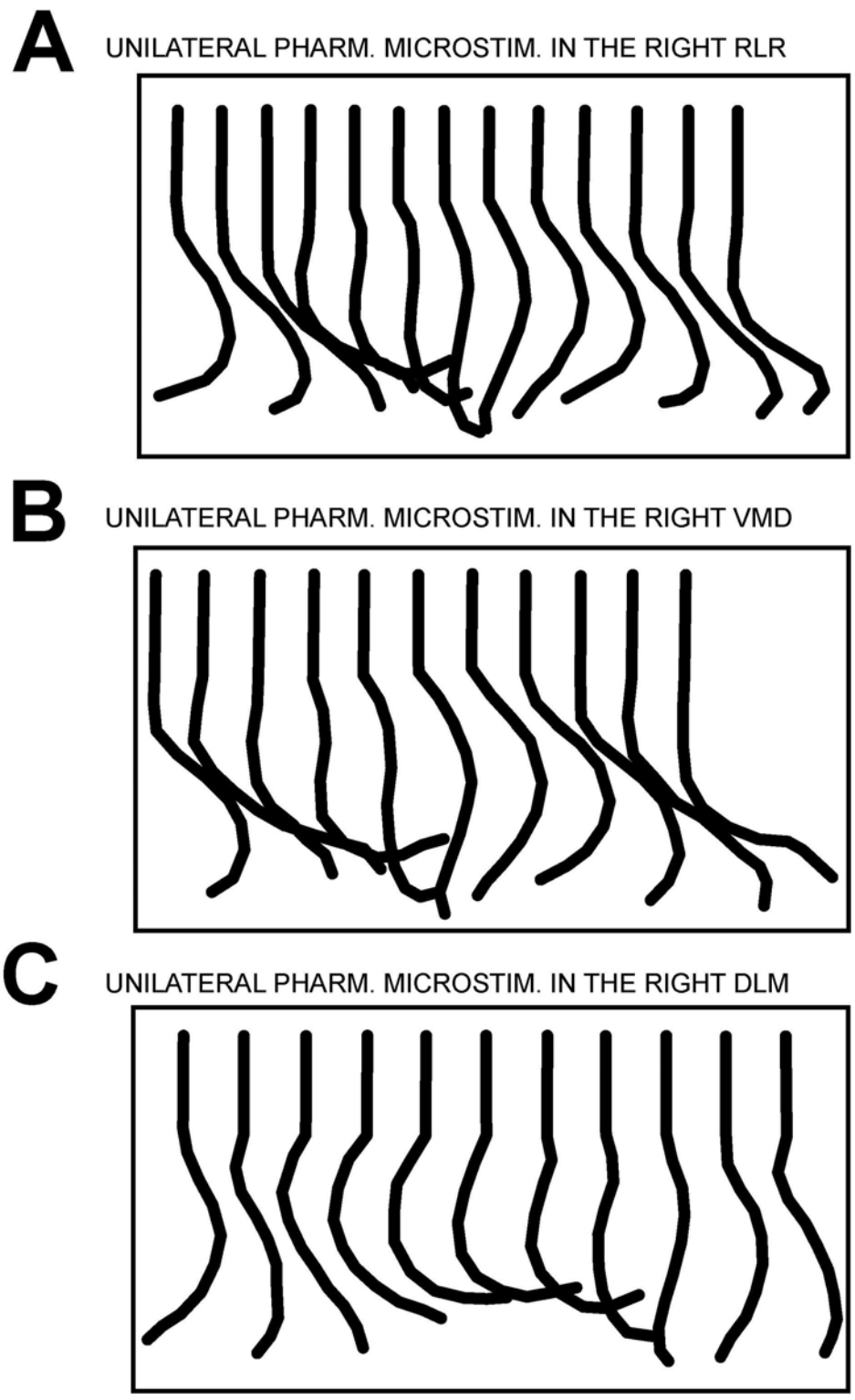
A
UNILATERAL PHARM. MICROSTIM. IN THE RIGHT RLR
B
UNILATERAL PHARM. MICROSTIM. IN THE RIGHT VMD
C
UNILATERAL PHARM. MICROSTIM. IN THE RIGHT DLM

Figure 10

Figure 11. Sequential symmetrical swimming movements, left to right, initiated by bilateral pharmacological microstimulation in the (A) aARRN (T = 363 ms, IFI = 33 ms), (B) pMRRN (T = 990 ms, IFI = 100 ms), and (C) mPRRN (T = 396 ms, IFI = 33 ms).

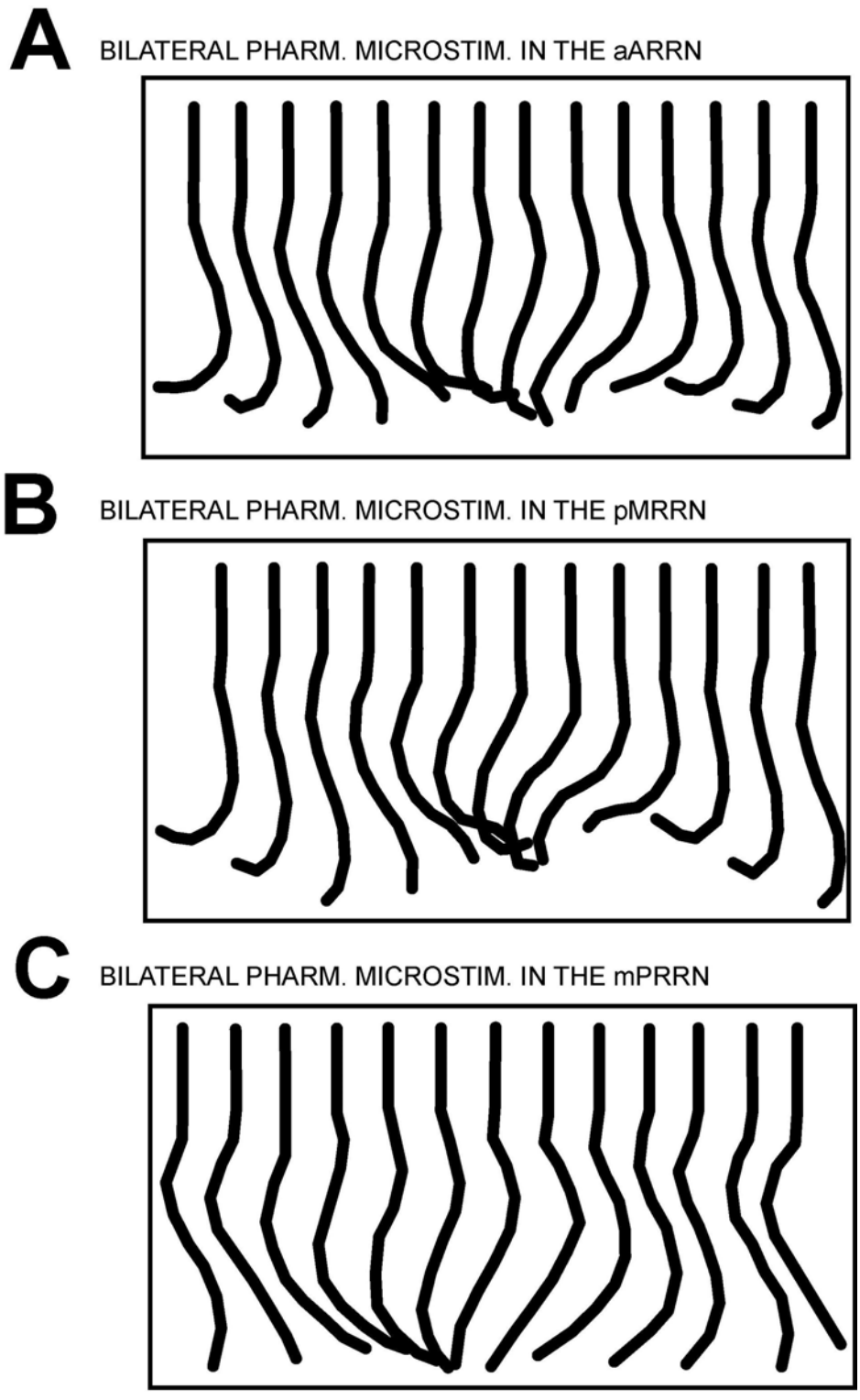
A
BILATERAL PHARM. MICROSTIM. IN THE aARRN
B
BILATERAL PHARM. MICROSTIM. IN THE pMRRN
C
BILATERAL PHARM. MICROSTIM. IN THE mPRRN

Figure 11

Figure 12. Normalized maximum lateral displacement versus normalized distance from the head during swimming movements in semi-intact preparations initiated by bilateral pharmacological microstimulation in the (A) aARRN (see Fig. 3D), (B) pMRRN, (C) aPRRN, (D) mPRRN, and (E) pPRRN.

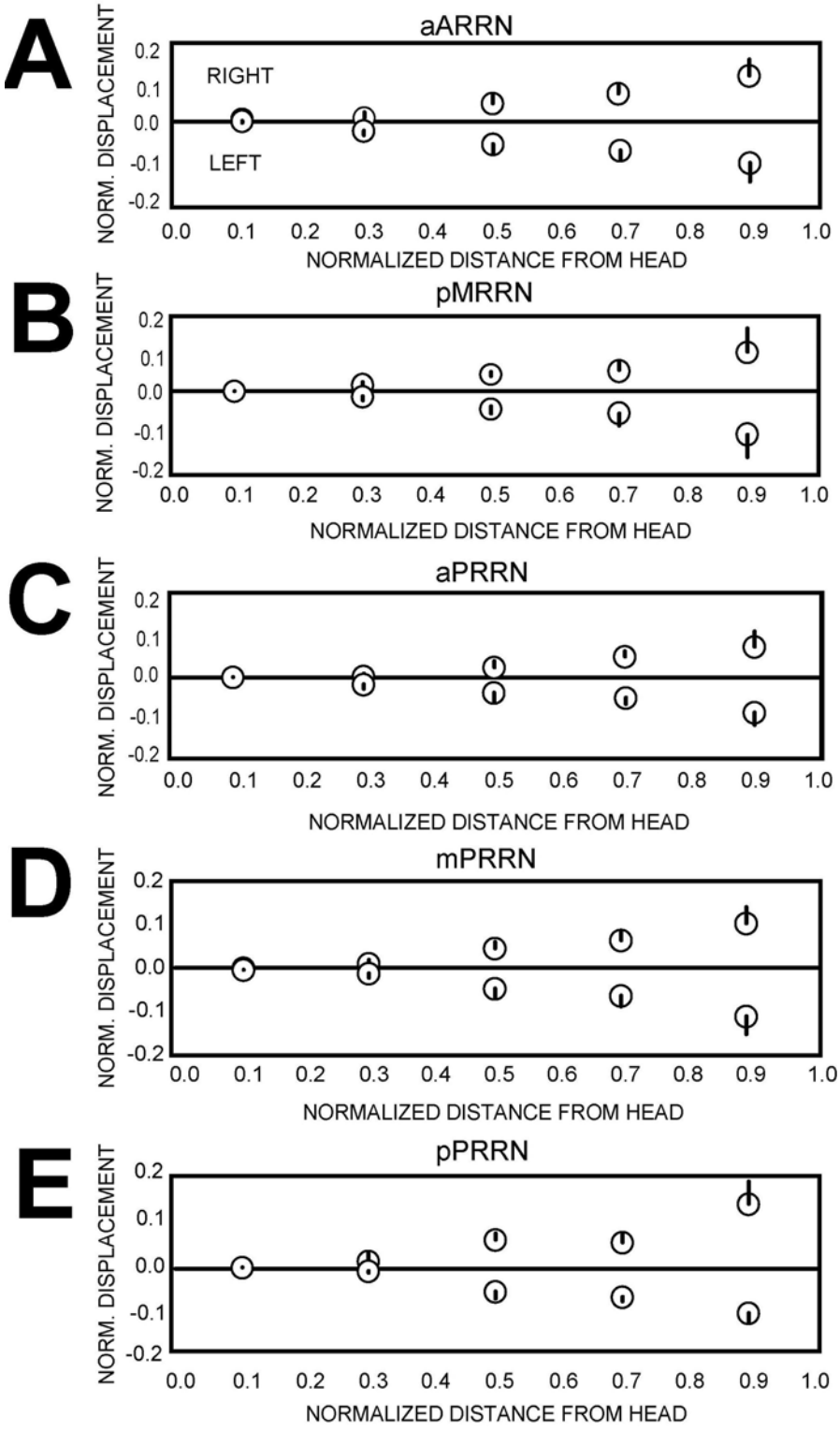
A
aARRN
RIGHT
LEFT
NORM. DISPLACEMENT
0.2
0.1
0.0
-0.1
-0.2
0.0 0.1 0.2 0.3 0.4 0.5 0.6 0.7 0.8 0.9 1.0
NORMALIZED DISTANCE FROM HEAD
B
pMRRN
NORM. DISPLACEMENT
NORMALIZED DISTANCE FROM HEAD
C
aPRRN
NORM. DISPLACEMENT
NORMALIZED DISTANCE FROM HEAD
D
mPRRN
NORM. DISPLACEMENT
NORMALIZED DISTANCE FROM HEAD
E
pPRRN
NORM. DISPLACEMENT
NORMALIZED DISTANCE FROM HEAD

Figure 12

Figure 13. (A) Bilateral pharmacological microstimulation in the aARRN. (A1) Brain diagram showing stimulation sites in the aARRN (filled squares). (A2) Stimulation in the aARRN initiated swimming movements and muscle activity, which consisted of left-right alternation of burst activity (1↔2) at the same segmental level (1,2 at 43% BL; see Fig. 2). (B) Bilateral pharmacological stimulation in the pMRRN. (B1) Diagram of brain showing stimulation sites in the pMRRN (filled squares). (B2) Alternating muscle burst activity similar to A2 but for stimulation in the pMRRN (1,2 at 45% BL).

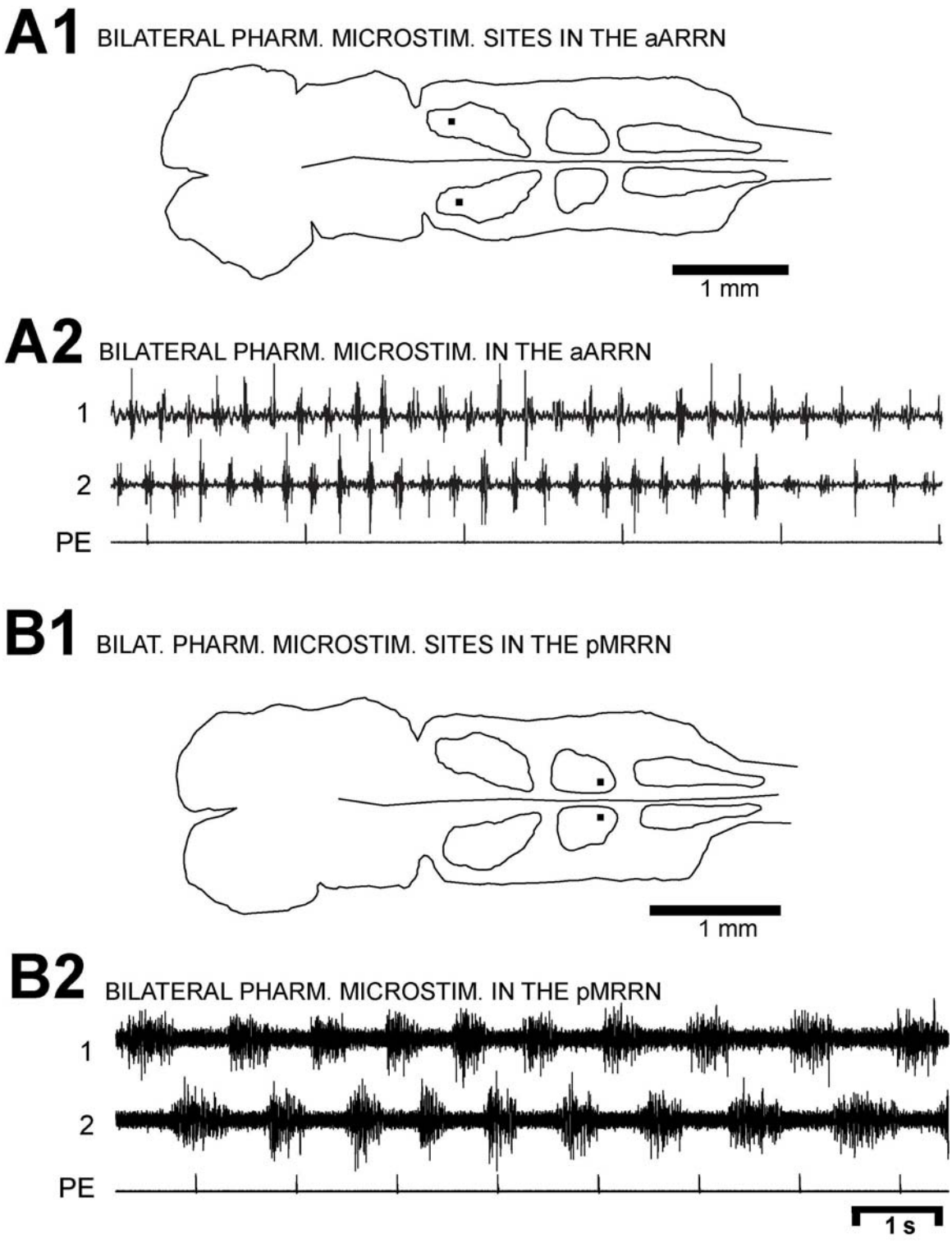
A1
BILATERAL PHARM. MICROSTIM. SITES IN THE aARRN
1 mm
A2
BILATERAL PHARM. MICROSTIM. IN THE aARRN
1
2
PE
B1
BILAT. PHARM. MICROSTIM. SITES IN THE pMRRN
1 mm
B2
BILATERAL PHARM. MICROSTIM. IN THE pMRRN
1
2
PE
1 s

Figure 13

Figure 14. (A) Bilateral pharmacological microstimulation in the aPRRN and mPRRN. (A1) Diagram of brain showing stimulation sites in the aPRRN and mPRRN (filled squares). (A2) Stimulation in the aPRRN initiated swimming muscle activity consisting of left-right alternation of burst activity (1↔2) at the same segmental level (1,2 at 43% BL). (A3) Left-right alternating swimming muscle activity initiated by stimulation in the mPRRN in the same animal as B (1,2 at 43% BL). (B) Bilateral stimulation in the pPRRN. (B1) Brain diagram showing stimulation sites in the pPRRN (filled squares). (B2) Alternating muscle activity recorded during an episode of swimming elicited by pharmacological stimulation in the pPRRN (1,2 at 47% BL).

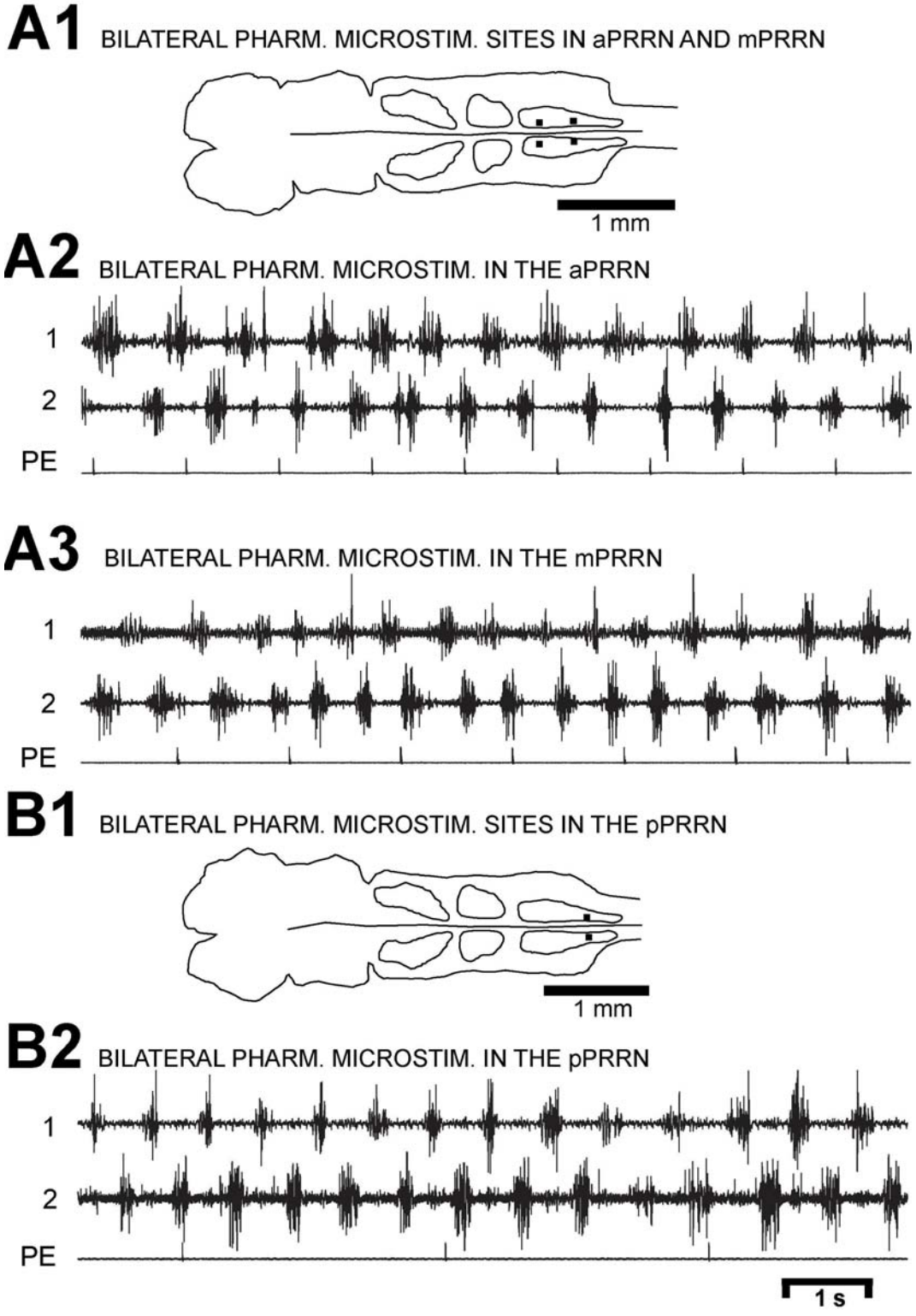
A1
BILATERAL PHARM. MICROSTIM. SITES IN aPRRN AND mPRRN
1 mm
A2
BILATERAL PHARM. MICROSTIM. IN THE aPRRN
1
2
PE
A3
BILATERAL PHARM. MICROSTIM. IN THE mPRRN
1
2
PE
B1
BILATERAL PHARM. MICROSTIM. SITES IN THE pPRRN
1 mm
B2
BILATERAL PHARM. MICROSTIM. IN THE pPRRN
1
2
PE
1 s

Figure 14

Figure 15. Sequential asymmetrical movements, left to right, elicited by unilateral pharmacological microstimulation in the (A) left aARRN (T = 528 ms, IFI = 33 ms), (B) right pMRRN (T = 528 ms, IFI = 33 ms), and (C) right aPRRN (T = 660 ms, IFI = 67 ms).

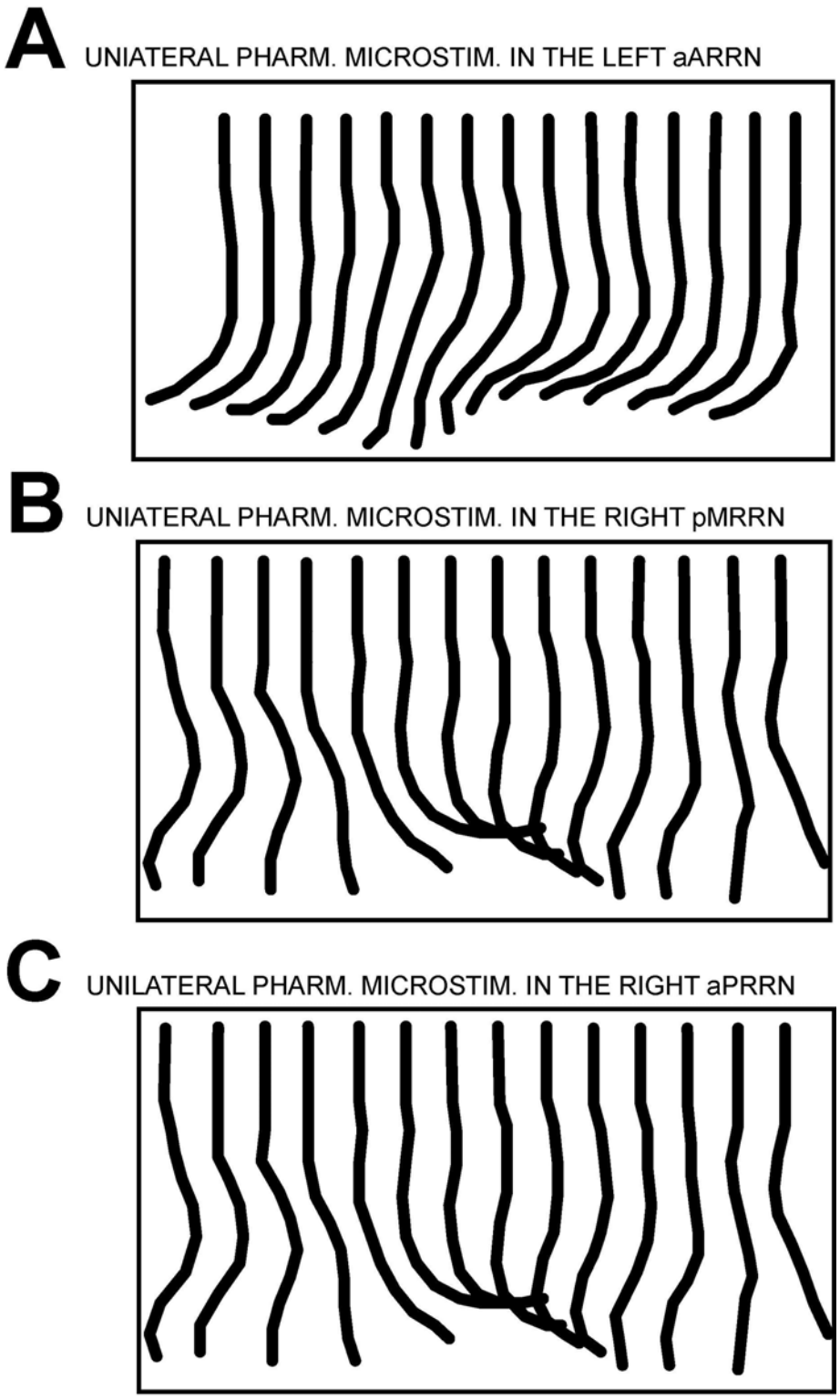
A
UNIATERAL PHARM. MICROSTIM. IN THE LEFT aARRN
B
UNIATERAL PHARM. MICROSTIM. IN THE RIGHT pMRRN
C
UNILATERAL PHARM. MICROSTIM. IN THE RIGHT aPRRN

Figure 15

Figure 16. (A) Schematic of brain showing traces around reticular nuclei (MRN, ARRN, MRRN, PRRN) and infundibulum (shaded ellipse) as well as bilaterally symmetrical pharmacological microstimulation sites in the VMD that initiated spinal locomotor activity (filled circles) as well as ineffective sites that did not elicit locomotor activity (open circles) (see text). (B) Enlargement of the brain and VMD locomotor area on the right side (left side omitted for simplicity) showing effective (C1-C3) and ineffective (C4-C6) stimulation sites for the matched recordings shown in C. (C) Spinal locomotor burst activity (1-3) and caudal spinal cord activity (SC; see Fig. 5A) initiated by pharmacological microstimulation (PE = pressure ejection pulses) within the VMD (C1-C3; filled circles in B), and uncoordinated burst activity initiated by stimulation in sites outside the VMD (C4-C6; open circles in B).

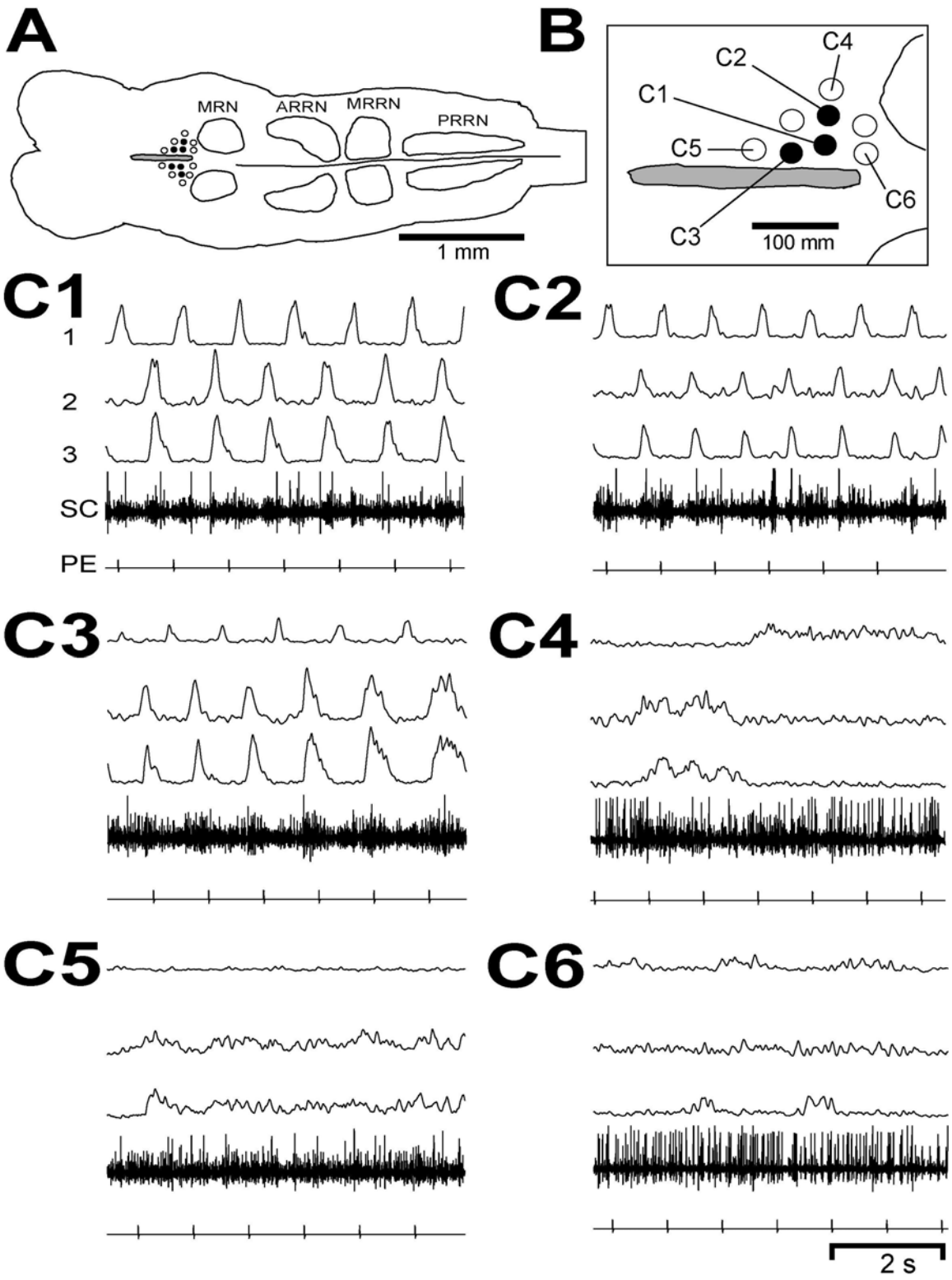
A
MRN
ARRN
MRRN
PRRN
1 mm
B
C4
C2
C1
C5
C6
C3
100 mm
C1
1
2
3
SC
PE
C2
C3
C4
C5
C6
2 s

Figure 16

Figure 17. (A) Diagram of brain showing reticular nuclei and bilateral pharmacological stimulation sites in the VMD (squares). (B) Spinal locomotor activity (1-3) and caudal spinal cord activity (SC; see Fig. 5A) initiated by pharmacological microstimulation (PE) in the VMD with (B1) 5 mM D-glutamate/5 mM D-aspartate, (B2) 0.5 mM NMDA, (B3) 1.0 mM AMPA, and (B4) 0.25 mM KA. Spinal locomotor activity consisted of left-right alternation of burst activity at the same segmental level (1↔2) and a rostrocaudal phase lag (2→3).

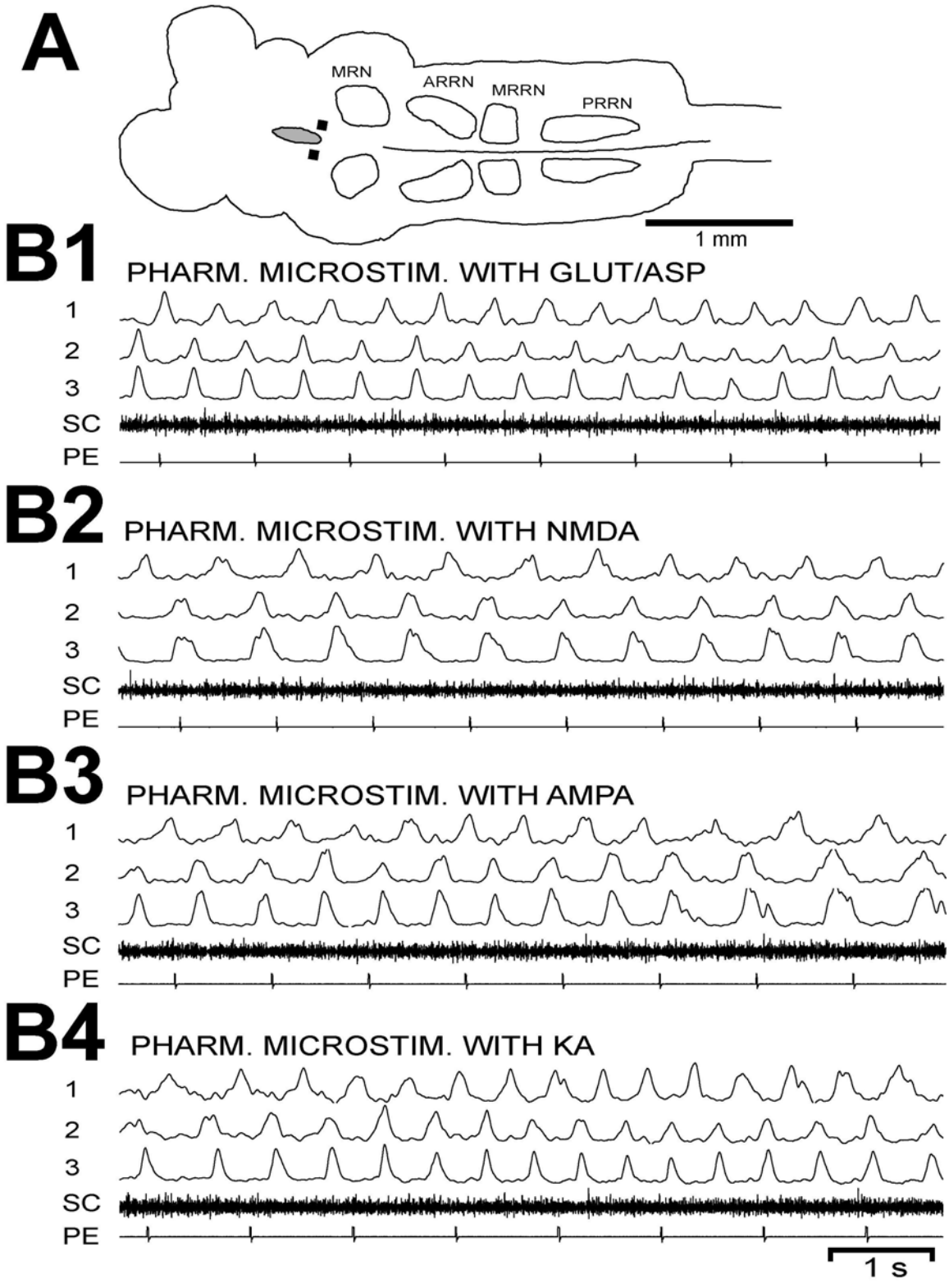
A
MRN
ARRN
MRRN
PRRN
1 mm
B1
PHARM. MICROSTIM. WITH GLUT/ASP
1
2
3
SC
PE
B2
PHARM. MICROSTIM. WITH NMDA
1
2
3
SC
PE
B3
PHARM. MICROSTIM. WITH AMPA
1
2
3
SC
PE
B4
PHARM. MICROSTIM. WITH KA
1
2
3
SC
PE
1 s

Figure 17

Figure 18. (A) Brain diagram showing bilaterally symmetrical microstimulation sites in the DLM (see legend in Fig. 16). (B) Enlarged view of the left side of the brain showing stimulation sites within the DLM that initiated spinal locomotor activity (filled circles, C1-C3) and sites just outside the DLM that were ineffective (open circles, C4-C6) for recordings shown in C. (C) Spinal locomotor activity (1-3) and caudal spinal cord activity (SC) elicited by pharmacological microstimulation within the DLM (C1-C3; filled circles in B), and uncoordinated burst activity elicited from sites outside the DLM (C4-C6; open circles in B). See legend in Fig. 16.

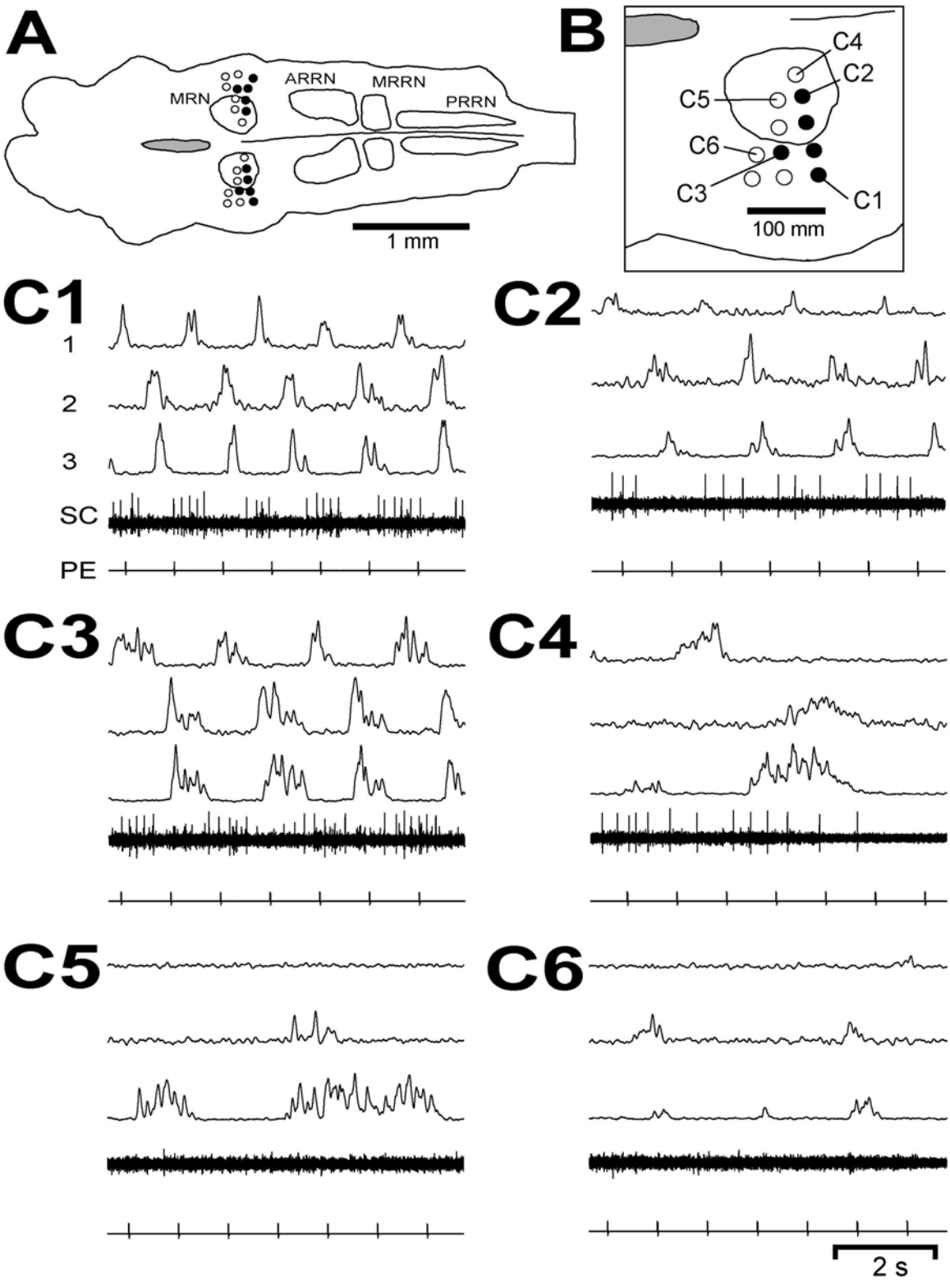
A
MRN
ARRN
MRRN
PRRN
1 mm
B
C4
C2
C5
C6
C3
C1
100 mm
C1
1
2
3
SC
PE
C2
C3
C4
C5
C6
2 s

Figure 18

Figure 19. (A) Diagram of brain showing reticular nuclei and bilateral stimulation sites in the DLM (squares). (B) Locomotor activity initiated by stimulation in the DLM with (B1) D-glutamate/D-asparate, (B2) NMDA, (B3) AMPA, and (B4) KA. See legend in Figure 17.

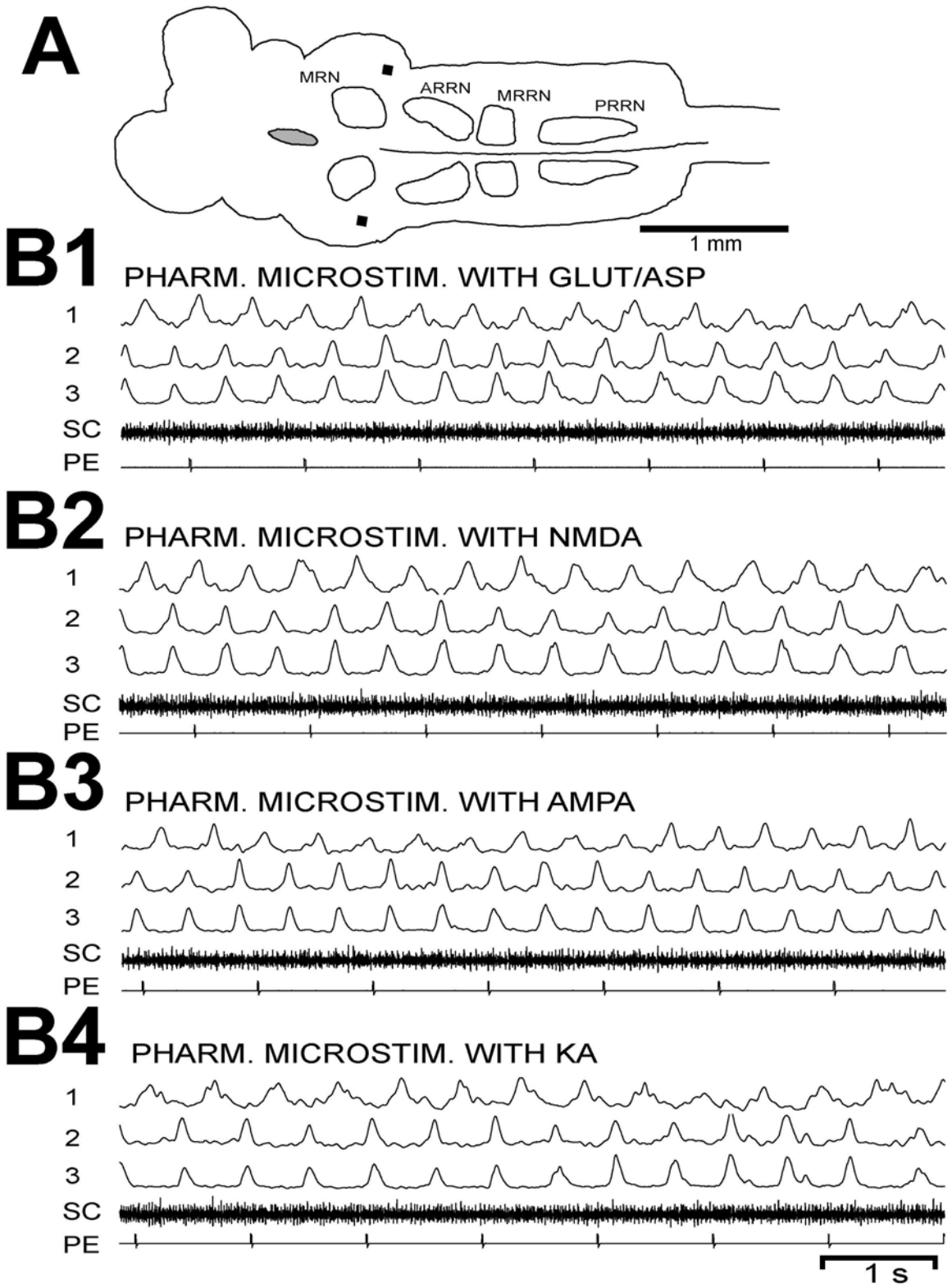
A
MRN
ARRN
MRRN
PRRN
1 mm
B1
PHARM. MICROSTIM. WITH GLUT/ASP
1
2
3
SC
PE
B2
PHARM. MICROSTIM. WITH NMDA
1
2
3
SC
PE
B3
PHARM. MICROSTIM. WITH AMPA
1
2
3
SC
PE
B4
PHARM. MICROSTIM. WITH KA
1
2
3
SC
PE
1 s

Figure 19

Figure 20. (A) Diagram of brain showing reticular nuclei (MRN, ARRN, MRRN, PRRN) and descending trigeminal tracts (dV; see Fig. 4A2) as well as stimulation sites in and around the RLR (see legend in Fig. 16). (B) Enlargement of left side of brain showing stimulation sites within the RLR that initiated spinal locomotor activity (filled circles, C1-C2) as well as sites outside the RLR that elicited uncoordinated activity (open circles, C3-C6) for the matched recordings shown in C. (C) Spinal locomotor activity initiated by pharmacological microstimulation within the RLR (C1-C2; filled circles in B), and uncoordinated activity elicited from sites outside the VMD (C3-C6; open circles in B). See legend in Figure 16.

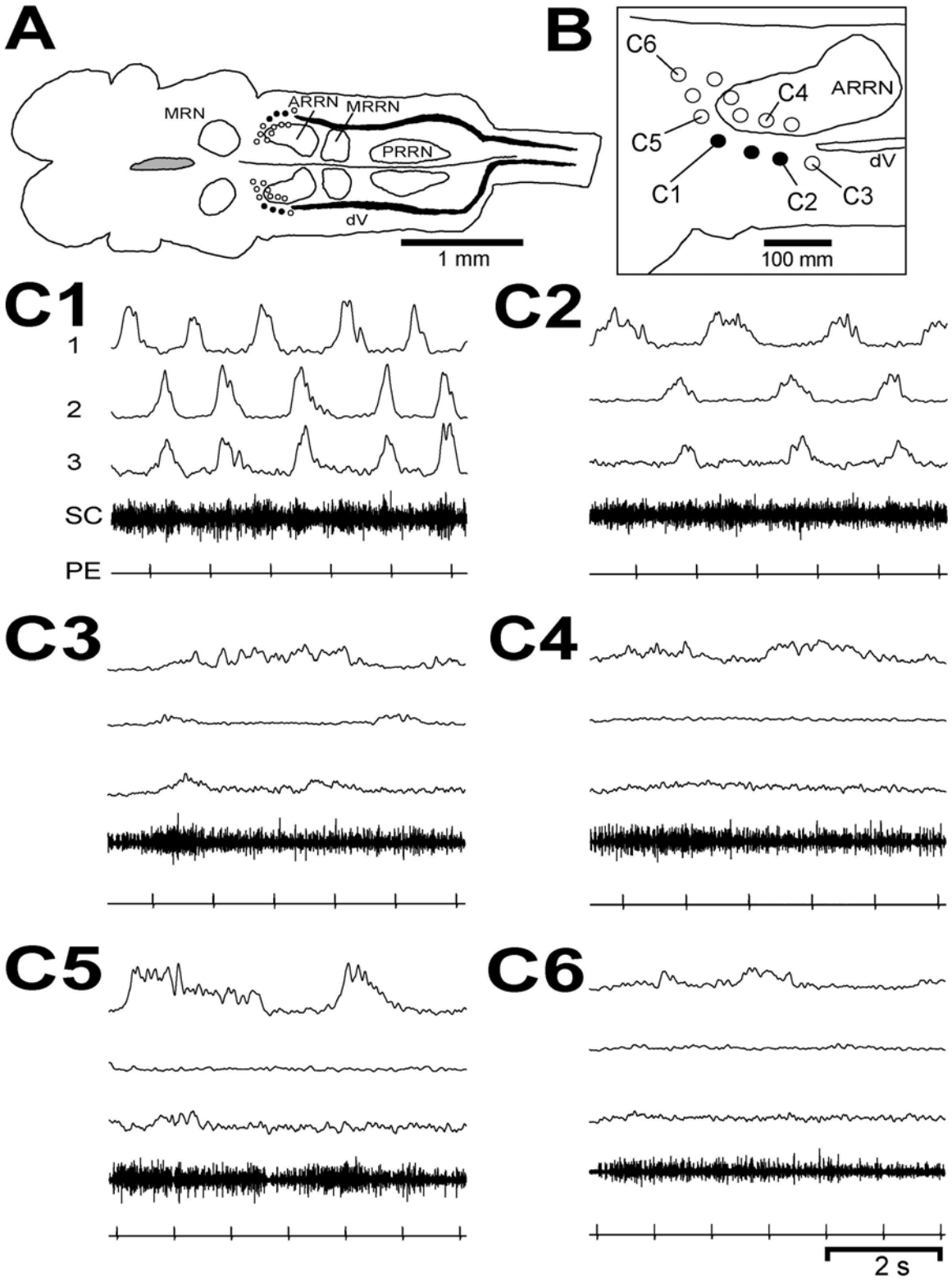
A
MRN
ARRN
MRRN
PRRN
dV
1 mm
B
C6
C4
ARRN
C5
dV
C1
C2
C3
100 mm
C1
1
2
3
SC
PE
C2
C3
C4
C5
C6
2 s

Figure 20

Figure 21. (A) Brain diagram showing reticular nuclei (MRN, ARRN, MRRN, and PRRN) and descending trigeminal tracts (dV) as well as bilateral stimulation sites in the RLR (triangles). (B) Locomotor activity initiated by stimulation in the RLR with (B1) D-glutamate/D-asparate, (B2) NMDA, (B3) AMPA, and (B4) KA. See legend in Figure 17.

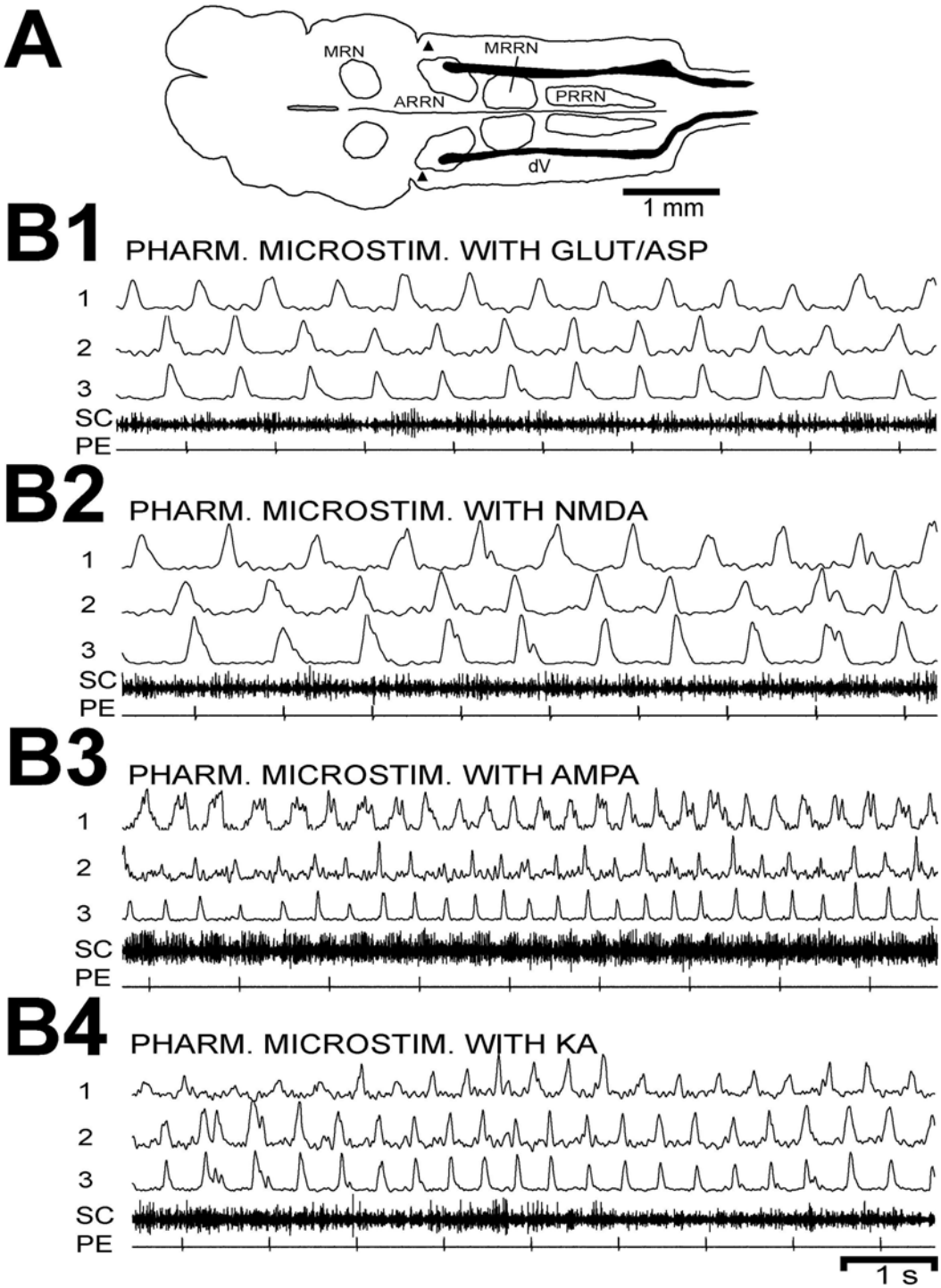
A
MRN
MRRN
ARRN
PRRN
dV
1 mm
B1
PHARM. MICROSTIM. WITH GLUT/ASP
1
2
3
SC
PE
B2
PHARM. MICROSTIM. WITH NMDA
1
2
3
SC
PE
B3
PHARM. MICROSTIM. WITH AMPA
1
2
3
SC
PE
B4
PHARM. MICROSTIM. WITH KA
1
2
3
SC
PE
1 s

Figure 21

Figure 22. (A) Diagram of partitioned *in vitro* brain/spinal cord preparation with bilaterally symmetrical pharmacological microstimulation pipettes (PE_R and PE_L), ventral root electrodes (1, 2, and 3), spinal cord electrode (SC), and Vaseline-sealed barrier (dark vertical line) creating a brain pool (Pool I) and spinal cord pool (Pool II). (B1-D1) Spinal locomotor activity (1-3) and caudal spinal cord activity (SC) initiated by microstimulation with D-glutamate/D-asparate in the VMD, DLM, or RLR consisting of left-right alternation of locomotor burst activity at the same segmental level ($1 \leftrightarrow 2$) and a rostrocaudal phase lag ($2 \rightarrow 3$). (B2-D2) After applying a zero calcium Ringer's solution to the brain (Pool I) to block chemical synaptic transmission, stimulation in the above higher locomotor areas no longer elicited ventral root burst activity (1-3) and elicited little or no activity in the caudal spinal cord (SC).

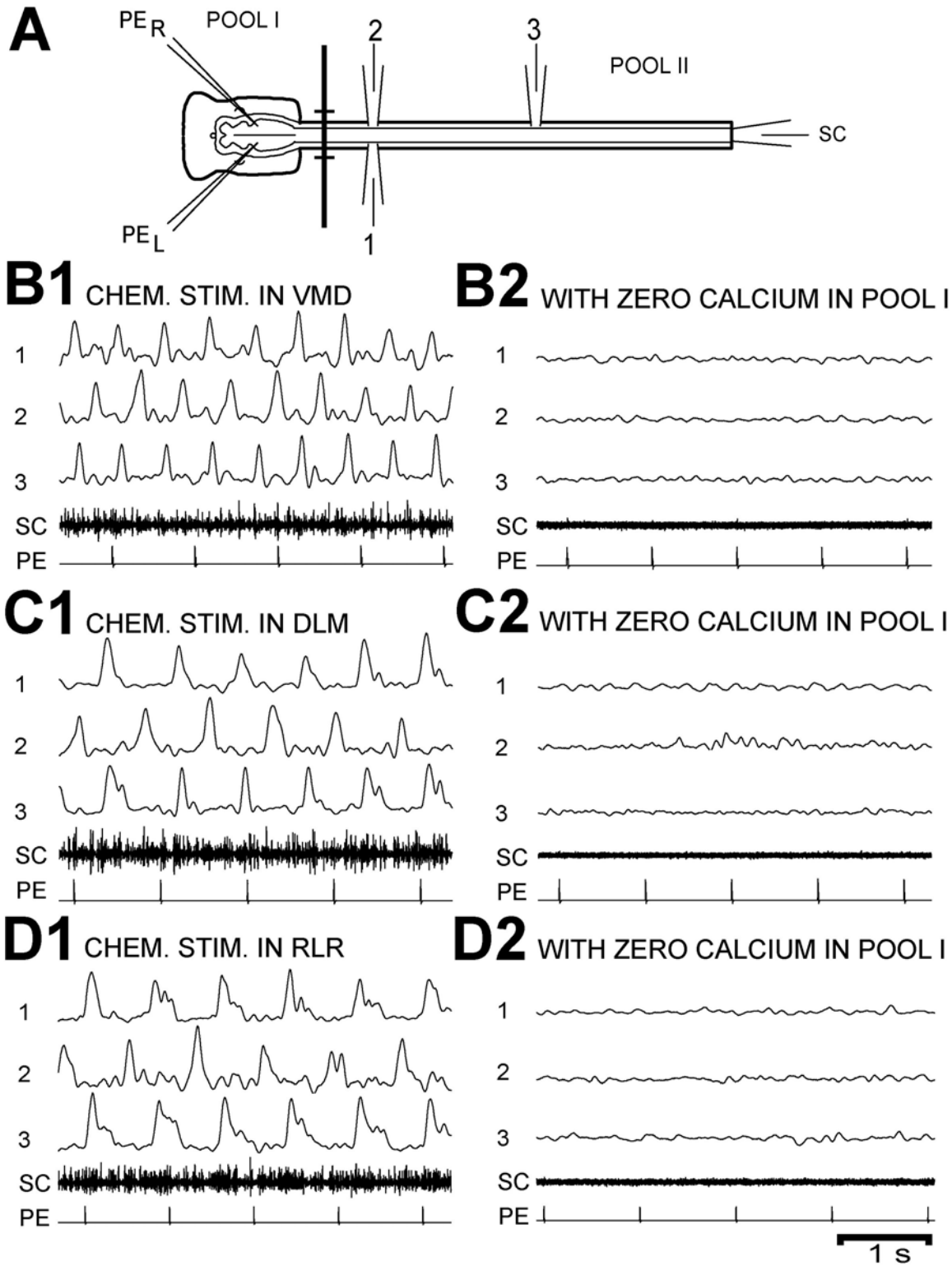
A
PE R
POOL I
2
3
POOL II
SC
PE L
1
B1 CHEM. STIM. IN VMD
B2 WITH ZERO CALCIUM IN POOL I
C1 CHEM. STIM. IN DLM
C2 WITH ZERO CALCIUM IN POOL I
D1 CHEM. STIM. IN RLR
D2 WITH ZERO CALCIUM IN POOL I
1
2
3
SC
PE
1 s

Figure 22

Figure 23. (A) Brain diagram with traces around reticular nuclei, bilateral stimulation sites in the RLR (triangles), and transection at the mesencephalic-rhombencephalic border (dark diagonal lines). (B) Prior to making a complete transection at the mesencephalic-rhombencephalic border. (B1) Stimulation in the RLR (PE) initiated well-coordinated spinal locomotor activity consisting of left-right alternation of burst acitivity (1→2) and a rostrocaudal phase lag (2→3). (B2) Brief mechanical stimulation of the left side of the oral hood (triangle) elicited some ventral root and caudal spinal cord activity. (C) Following a transection at the mesencephalic-rhombencephalic border (dark lines in A; same animal as B). (C1) Stimulation in the RLR (PE) no longer elicited locomotor activity but did produce some activity at the caudal end of the spinal cord (SC). (C2) Mechanical stimulation of the left side of the oral hood (triangle) still elicited some ventral root and caudal spinal cord activity.

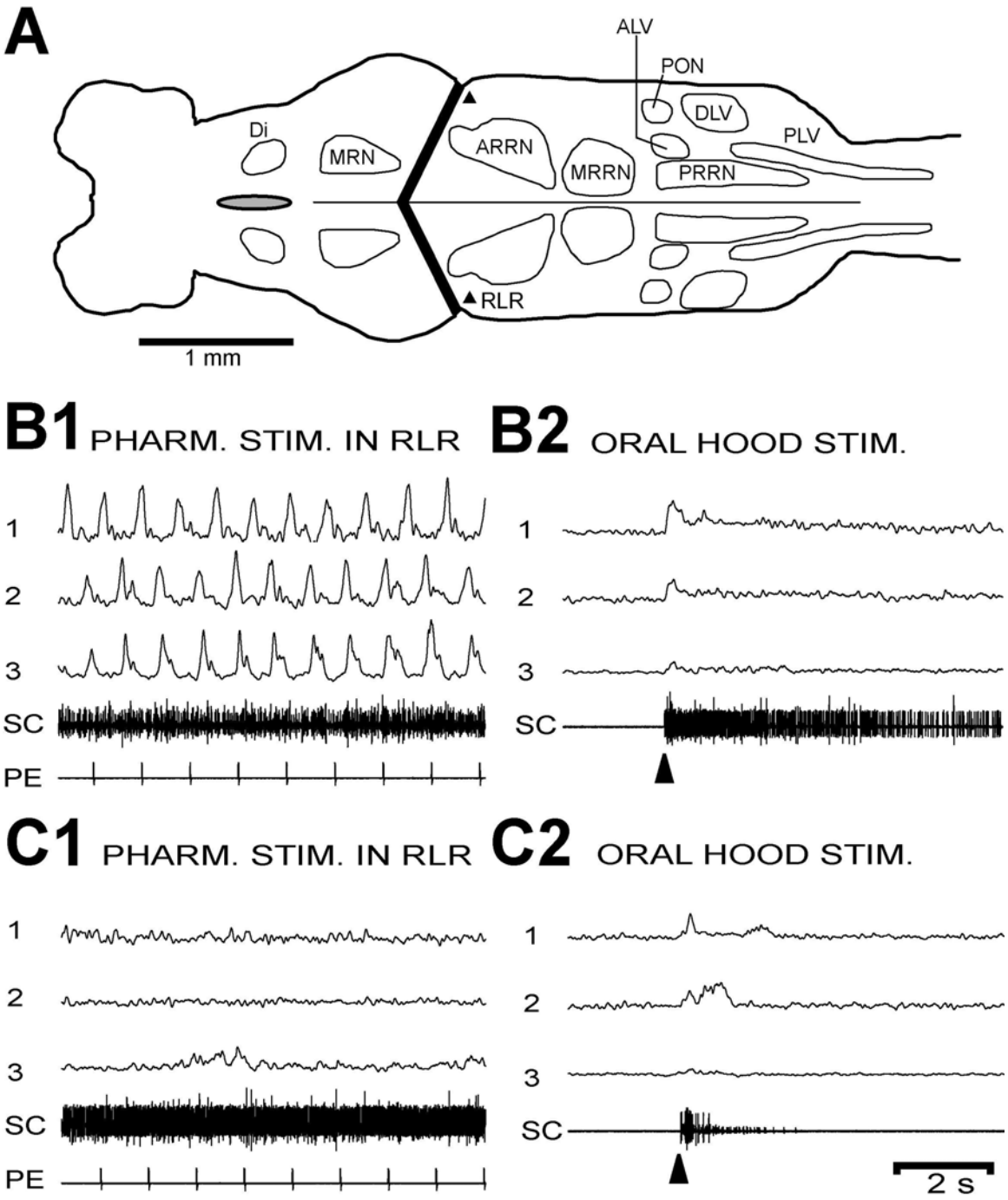
A
ALV
PON
DLV
PLV
Di
MRN
ARRN
MRRN
PRRN
RLR
1 mm
B1 PHARM. STIM. IN RLR
B2 ORAL HOOD STIM.
1
2
3
SC
PE
C1 PHARM. STIM. IN RLR
C2 ORAL HOOD STIM.
2 s

Figure 23

Figure 24. Disruption of left-right coupling between locomotor networks in the caudal spinal cord in whole animals. (A) Diagram of a whole animal showing electrodes for recording muscle activity (EMGs) at 20% body length (BL, normalized distance from head) (1,2) and 40% BL (3,4), midline lesion in the caudal spinal cord (thick horizontal line, 30%→50% BL), and spinal cord transection site (T; thick vertical line at 50% BL). (B) In an animal with a midline lesion in the caudal spinal cord but without (w/o) a spinal cord transection, brief electrical stimulation of the tail (applied prior to beginning of record) elicited escape swimming and locomotor muscle activity, which consisted of left-right alternation (1↔2 and 3↔4) and a rostrocaudal phase lag (2→3 and 1→4). (C) Different animal than "B" that had both a midline lesion in the caudal spinal cord and spinal cord transection at 50% BL. (C1) Sensory-evoked locomotor muscle activity was present in the rostral and caudal body. (C2,C3) Same animal as in "C1" showing short "burstlets" (*) that either were grouped together to form longer bursts (C2) or continuous during rostral burst activity (C3).

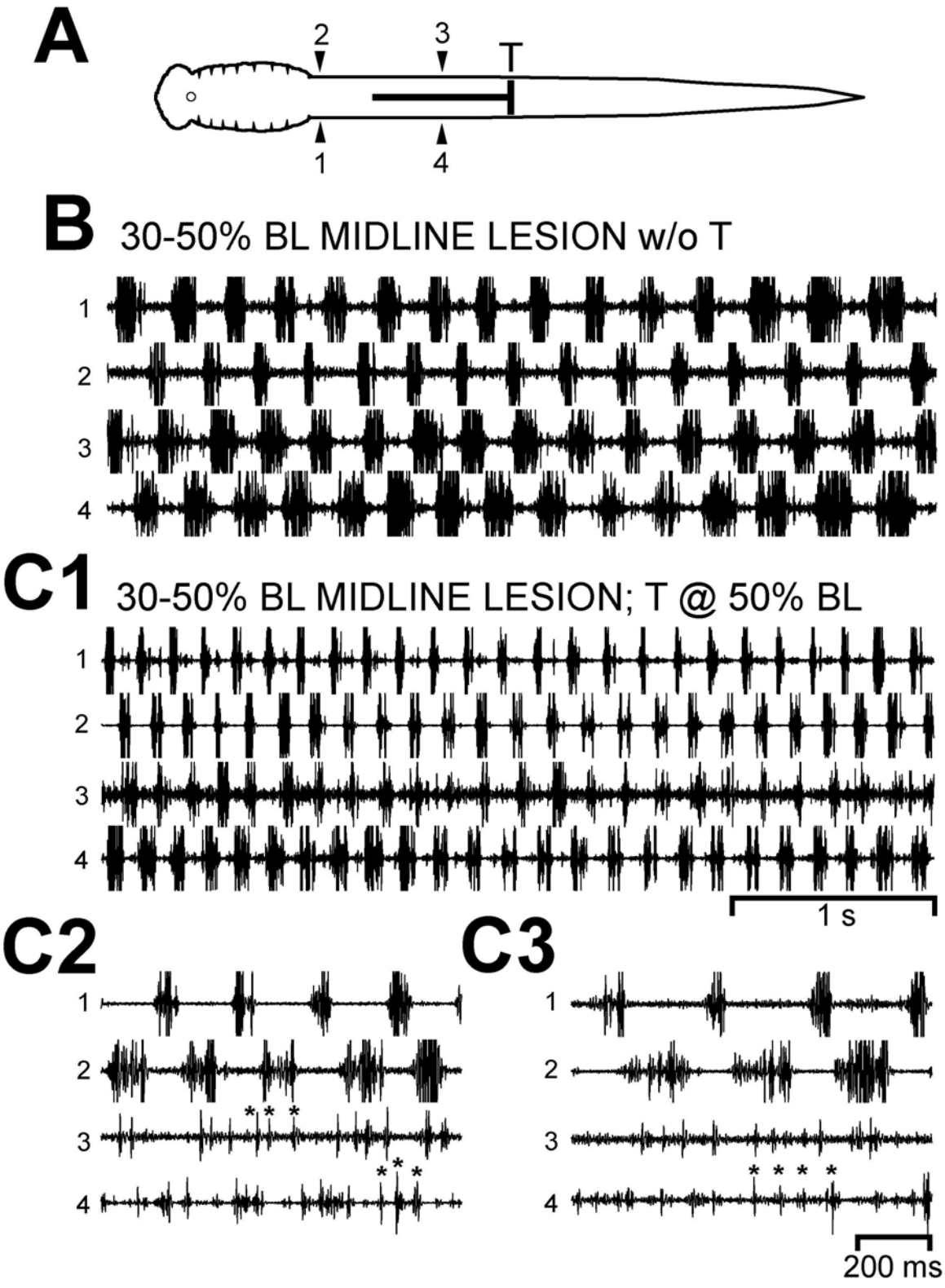
A
2
3
T
1
4
B
30-50% BL MIDLINE LESION w/o T
1
2
3
4
C1
30-50% BL MIDLINE LESION; T @ 50% BL
1
2
3
4
1 s
C2
1
2
3
4
C3
1
2
3
4
200 ms

Figure 24

Figure 25. *In vitro* spinal motor activity following disruption of left-right coupling between motor networks in the caudal spinal cord. (A) *In vitro* brain/spinal cord preparation (same preparation for B1-B3) showing pharmacological microstimulation pipettes (PE_L, PE_R), ventral root recording electrodes at 20% (1,2) and 40% BL (3,4), midline lesion in the caudal spinal cord (horizontal line, 30%→50% BL), and spinal cord transection site (T, vertical line at 50% BL). (B1) Prior to performing lesions, chemical microstimulation in brain locomotor areas (PE = pressure ejection pulses; see Methods) initiated well-coordinated locomotor activity consisting of left-right alternation (1↔2 and 3↔4) and a rostrocaudal phase lag (2→3 and 1→4). (B2) After a longitudinal midline lesion in the caudal cord, stimulation in the same brain locomotor areas initiated left-right alternating burst activity in the rostral, intact (1↔2) and caudal, lesioned (3↔4) spinal cord, but the activity usually was more erratic in the caudal cord. (B3) Following a spinal cord transection at 50% BL, stimulation in the brain initiated alternating burst activity in the rostral and caudal spinal cord. In B2/B3, the gains of channels 3 and 4 were increased by 1.25X and 2X, respectively, compared to B1.

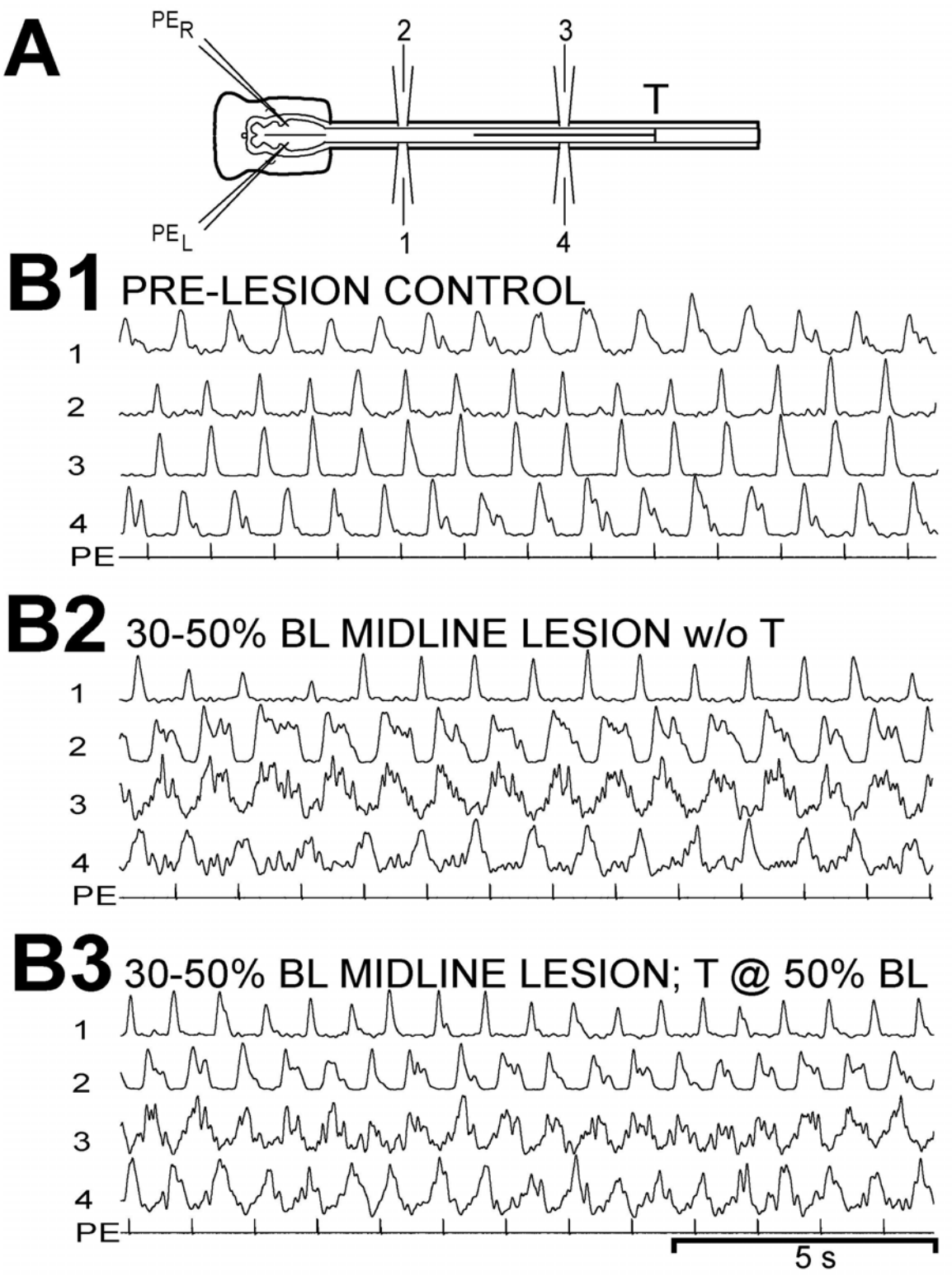
A
PE_R
2
3
T
PE_L
1
4
B1 PRE-LESION CONTROL
1
2
3
4
PE
B2 30-50% BL MIDLINE LESION w/o T
1
2
3
4
PE
B3 30-50% BL MIDLINE LESION; T @ 50% BL
1
2
3
4
PE
5 s

Figure 25

Figure 26. Experiment to test whether left and right caudal hemi-spinal cords are rhythmogenic in response to descending activation from the brain. (A) Partitioned i*n vitro* brain/spinal cord preparation showing brain pool (I), rostral and caudal spinal cord pools (II and III), pharmacological microstimulation pipettes (PE_L, PE_R), ventral root recording electrodes (1-4), and midline lesion in the caudal spinal cord (horizontal line, 30%→50% BL). (B1) With a midline lesion in the caudal spinal cord and normal Ringer's solution in all pools, pharmacological microstimulation in brain locomotor areas (PE) initiated left-right alternating burst activity in the rostral and caudal spinal cord (1↔2 and 3↔4). (B2) With a low calcium Ringer's solution applied to the rostral spinal cord (Pool II), stimulation in the same brain locomotor areas no longer elicited rhythmic burst activity in the caudal cord (3,4). (B3) After normal Ringer's solution was returned to the rostral spinal cord pool (Pool II), brain-evoked alternating burst activity was restored in the rostral and caudal spinal cord. (B4) Following the above procedures, the very rostral spinal cord was transected, and application of 1 mM D-glutamate to the spinal cord (pools II and III) elicited alternating burst activity only in the rostral, intact spinal cord (1↔2).

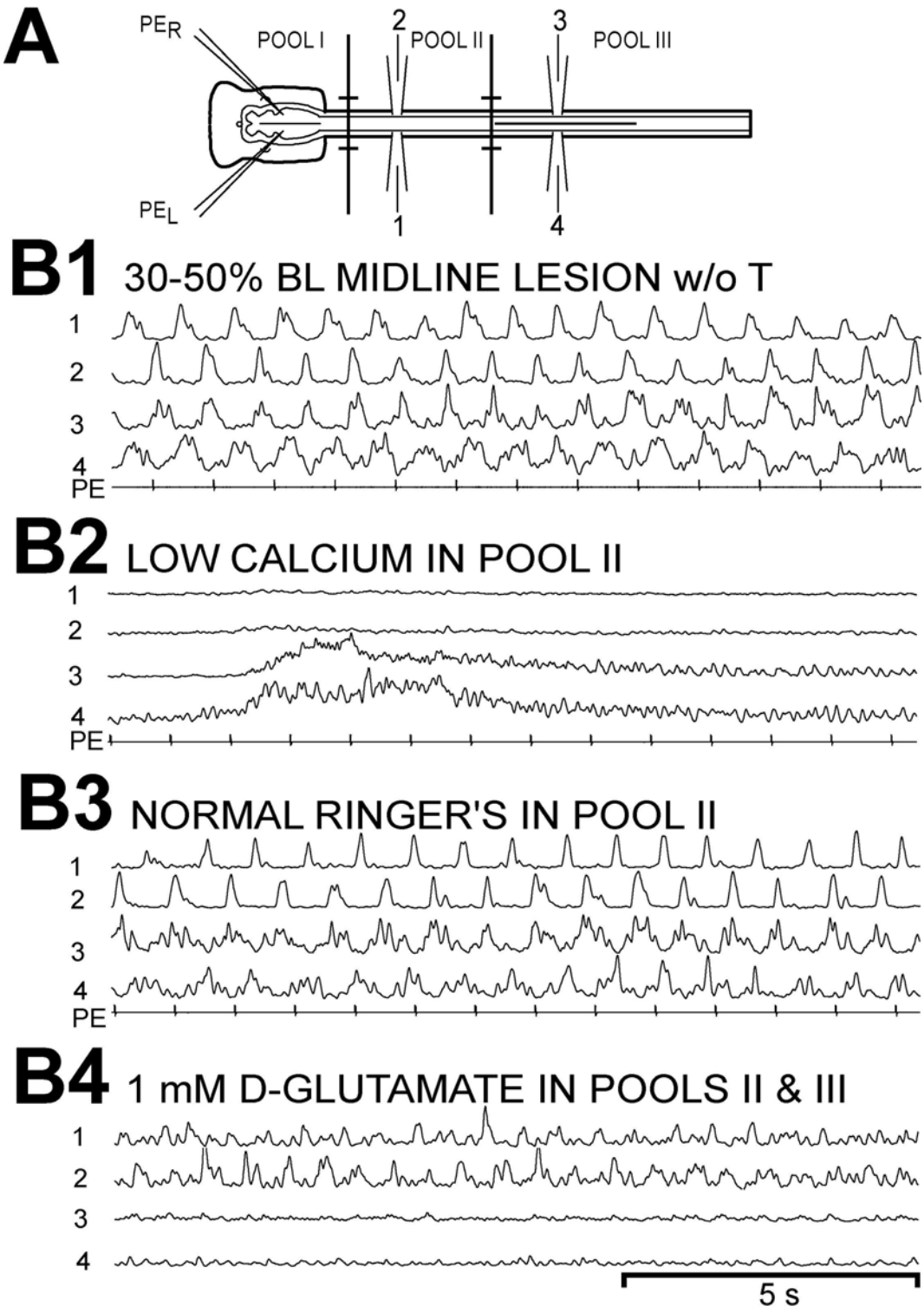
A
PE_R
POOL I
2
POOL II
3
POOL III
PE_L
1
4
B1 30-50% BL MIDLINE LESION w/o T
1
2
3
4
PE
B2 LOW CALCIUM IN POOL II
1
2
3
4
PE
B3 NORMAL RINGER'S IN POOL II
1
2
3
4
PE
B4 1 mM D-GLUTAMATE IN POOLS II & III
1
2
3
4
5 s

Figure 26

Figure 27. Disruption of left-right coupling between locomotor networks in the rostral spinal cord in whole animals. (A) Diagram of a whole animal showing muscle recording electrodes at 20% (1,2) and 40% BL (3,4), midline lesion in the rostral spinal cord (thick horizontal line, 8%→30% BL), and spinal cord transection site (T; thick vertical line at 30% BL). (B) Following a midline lesion in the rostral spinal cord (w/o spinal cord transection), brief electrical stimulation of the oral hood (applied prior to beginning of record) initiated left-right alternating muscle burst activity in both the rostral (1↔2) and caudal (3↔4) body. (C) In a different animal than "B" with both a midline lesion in the rostral spinal cord as well as a spinal cord transection at 30% BL, stimulation of the oral hood (arrowhead) elicited uncoordinated muscle activity in the rostral body (1,2), but undulatory or locomotor-like movements were never observed. In "C", the gains of channels 1 and 2 were lowered substantially to more clearly reveal the burst activity.

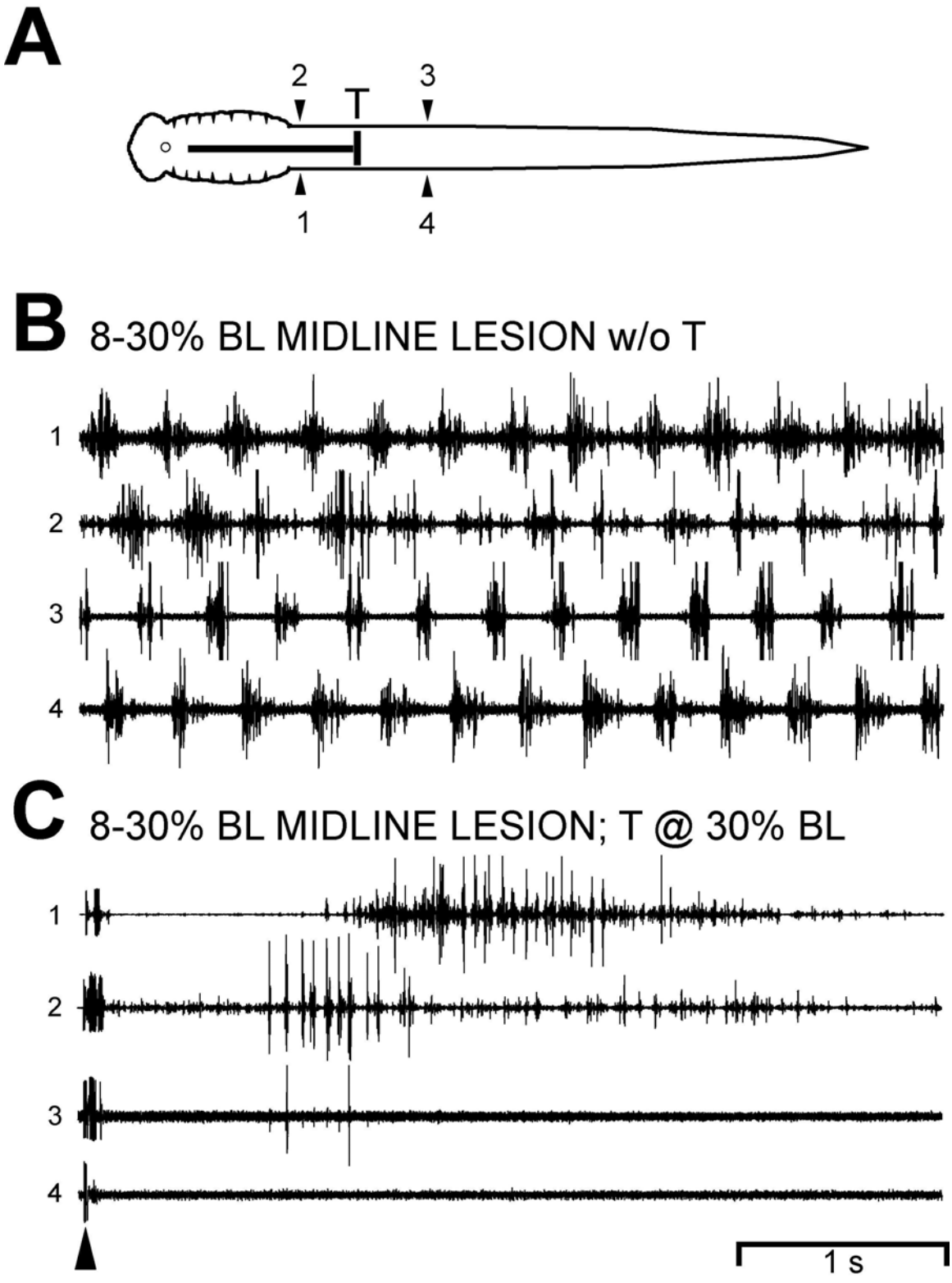
A
2
T
3
1
4
B
8-30% BL MIDLINE LESION w/o T
1
2
3
4
C
8-30% BL MIDLINE LESION; T @ 30% BL
1
2
3
4
1 s

Figure 27

Figure 28. Motor activity following disruption of left-right coupling in the rostral spinal cord before and after a spinal cord transection at 30% BL in the same animal. (A) Diagram of a whole animal showing muscle recording electrodes (1-4, see Fig. 27), midline lesion in the rostral spinal cord (horizontal line, 8%→30% BL), and spinal cord transection site (T; vertical line at 30% BL). Same animal for all recordings. (B1) Following a rostral midline lesion (w/o spinal cord transection), left-right alternating muscle burst activity was present in the rostral and caudal body. (B2) Following a spinal cord transection at 30% BL, brief electrical stimulation of the oral hood (arrowhead) elicited uncoordinated muscle activity in the rostral body (1,2), but undulatory or locomotor-like movements were never observed. In "B2", the gains of channels 1, 2 were lowered and those of 3, 4 were increased relative to "B1" to better reveal the burst activity. (B3) Recordings following spinal cord transection at 30% BL showing occasional weak alternating rostral "bursts" composed of relatively short "burstlets" (*; see text). Activity in B3ii is during thick bar in B3i.

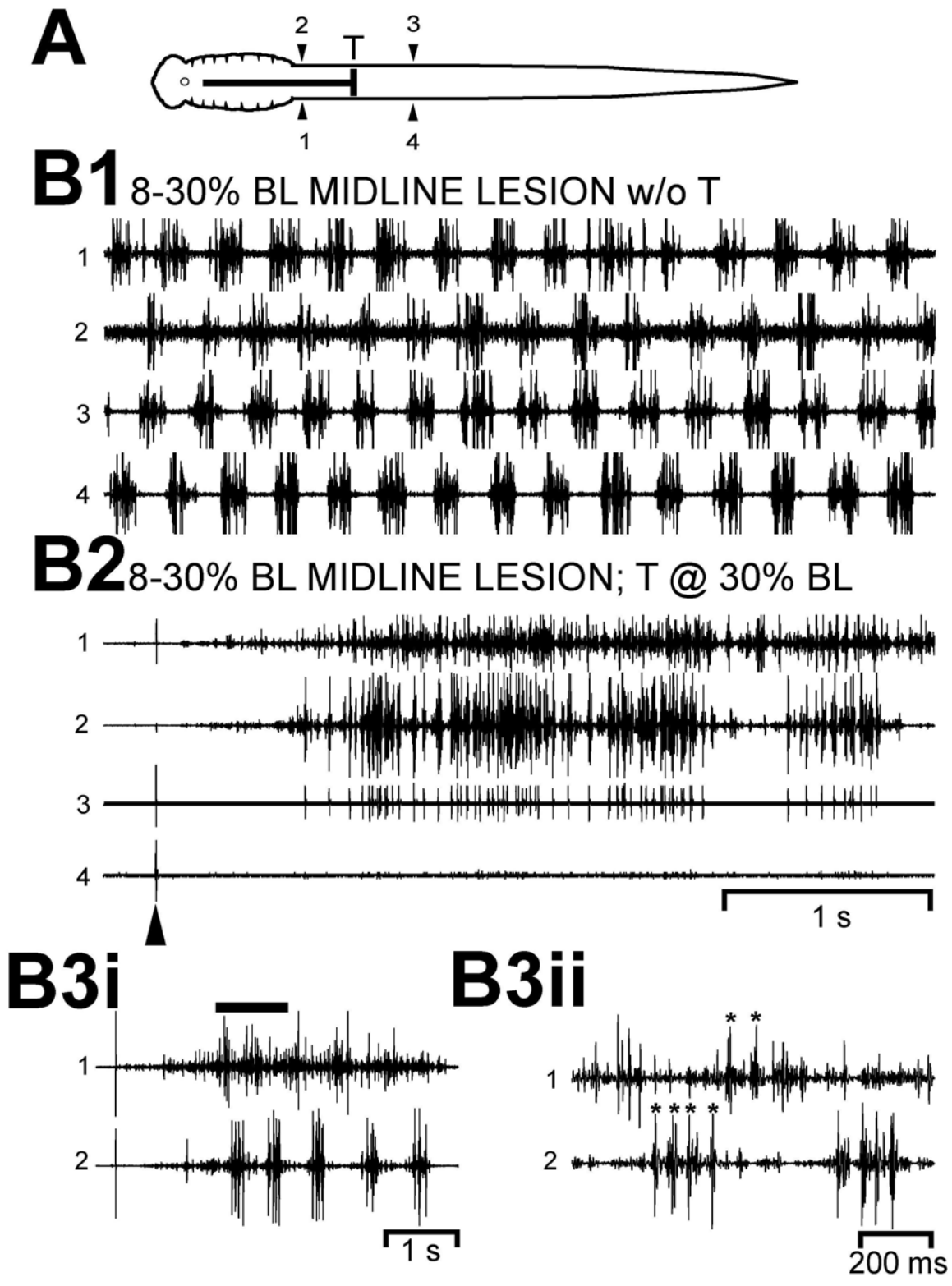
A
2
T
3
1
4
B1 8-30% BL MIDLINE LESION w/o T
1
2
3
4
B2 8-30% BL MIDLINE LESION; T @ 30% BL
1
2
3
4
1 s
B3i
1
2
1 s
B3ii
1
2
200 ms

Figure 28

Figure 29. Spinal motor activity from an *in vitro* preparation following disruption of left-right coupling between locomotor networks in the rostral spinal cord. (A) *In vitro* brain/spinal cord preparation (see Fig. 25) showing midline lesion in the rostral spinal cord (horizontal line, 8%→30% BL), and spinal cord transection site (T; vertical line at 30% BL). (B1) Prior to performing lesions, stimulation in brain locomotor areas (PE) initiated well-coordinated *in vitro* locomotor activity (1-4). (B2) Following a midline lesion in the rostral spinal cord, left-right alternating burst activity was present in caudal (3↔4) but not rostral (1,2) spinal ventral roots. (B3) Following a spinal cord transection at 30% BL, stimulation in brain locomotor areas elicited uncoordinated ventral root activity in the rostral cord (1,2) (however, see Figs. 30,31). In B2 and B3, the gains of channels 3 and 4 were increased by 2X relative to B1. Scale bar = (B1,B2) 5 s; (B3) 16 s.

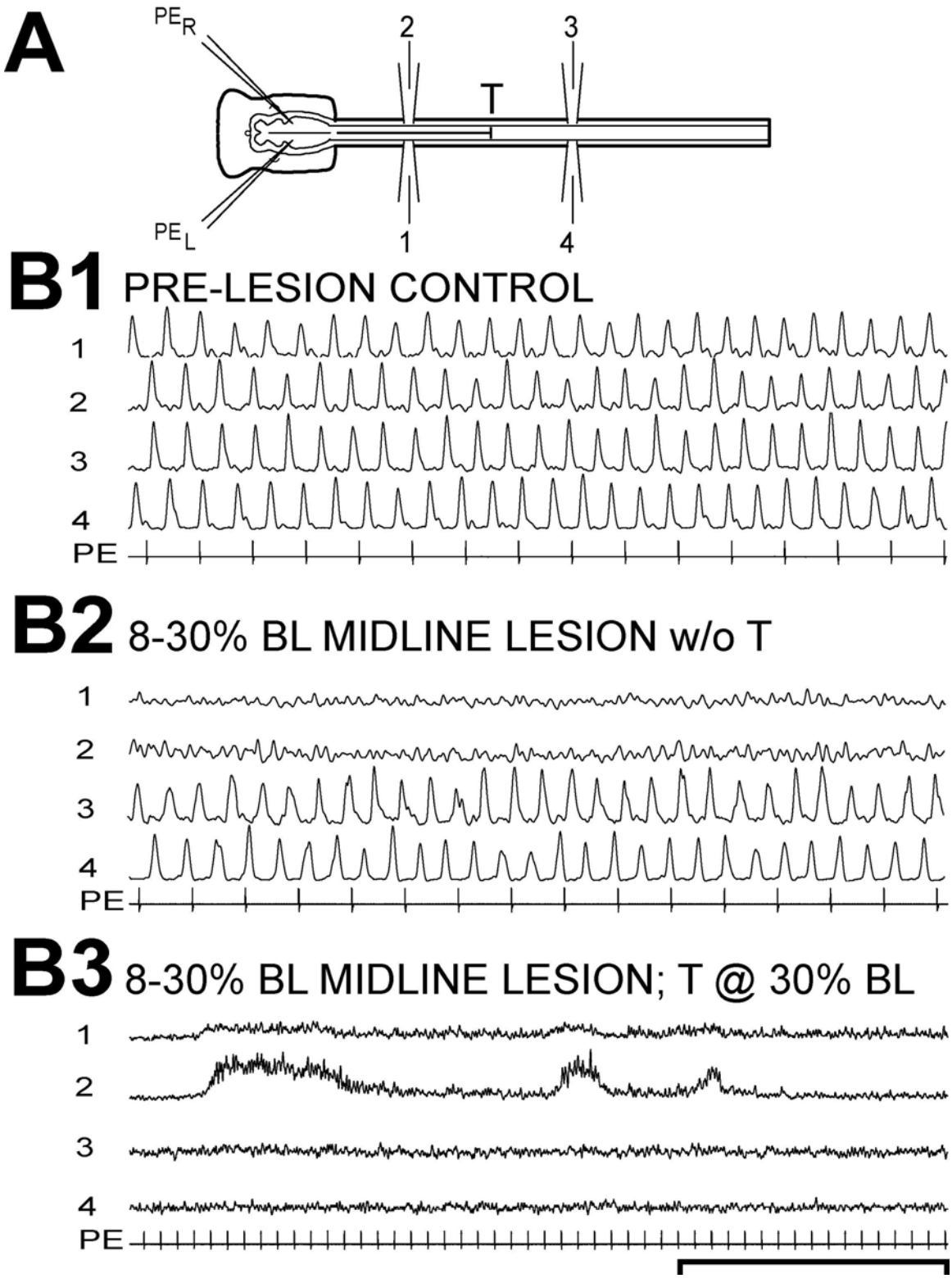
A
PE R
PE L
1
2
3
4
T
B1 PRE-LESION CONTROL
1
2
3
4
PE
B2 8-30% BL MIDLINE LESION w/o T
1
2
3
4
PE
B3 8-30% BL MIDLINE LESION; T @ 30% BL
1
2
3
4
PE

Figure 29

Figure 30. *In vitro* motor activity following disruption of left-right coupling in the rostral spinal cord. (A) *In vitro* brain/spinal cord preparation (see Fig. 29) showing midline lesion in the rostral spinal cord (horizontal line, 8%→30% BL), and spinal cord transection site (T; vertical line at 30% BL). (B1) Prior to performing lesions, stimulation in brain locomotor areas initiated *in vitro* locomotor activity (1-4). (B2) Following a rostral midline lesion, left-right burst activity was present in caudal (3↔4) but not rostral (1,2) ventral roots. (B3) Following a spinal cord transection at 30% BL, stimulation in brain locomotor areas elicited relatively slow alternating ventral root activity in the rostral spinal cord (1↔2). In B2 (B3), the gains of channels 1, 2, 3, and 4 were increased by 4X, 2X, 2X, and 2X (2X, 2X, 1X, and 2X), respectively, relative to B1. Scale bar = (B1,B2) 5 s; (B3) 16 s.

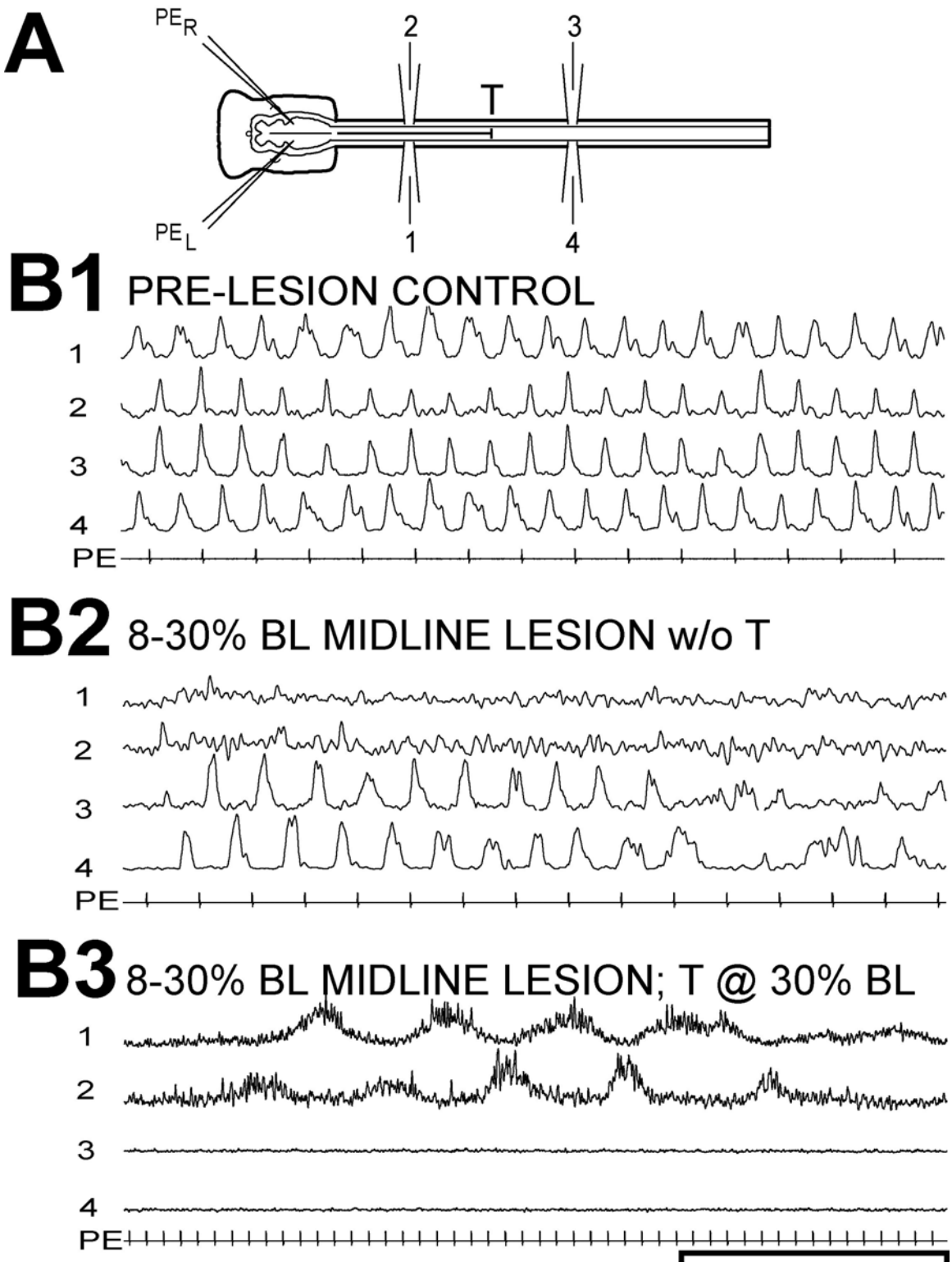
A
PE_R
2
3
T
PE_L
1
4
B1 PRE-LESION CONTROL
1
2
3
4
PE
B2 8-30% BL MIDLINE LESION w/o T
1
2
3
4
PE
B3 8-30% BL MIDLINE LESION; T @ 30% BL
1
2
3
4
PE

Figure 30

Figure 31. (A) Partitioned *in vitro* brain/spinal cord preparation showing brain pool (I), spinal cord pool (II), pharmacological microstimulation pipettes, ventral root electrodes (20% and 30% BL), midline lesion in the rostral spinal cord (horizontal line, 8%→40% BL), and spinal cord transection (T) at 40% BL (n = 4). (B1) Following a spinal cord transection at 40% BL but prior to performing a midline lesion, stimulation in brain locomotor areas initiated well-coordinated *in vitro* locomotor activity (1-3). (B2) Following a midline lesion in the rostral spinal cord, stimulation in the brain elicited rhythmic burst activity consisting of relatively slow alternation (1↔2) and nearly synchronous ipsilateral burst activity (2 – 3). In B2, the gains of channels 1, 2, and 3 were increased by 2X, 2X, and 5X, respectively, relative to B1.

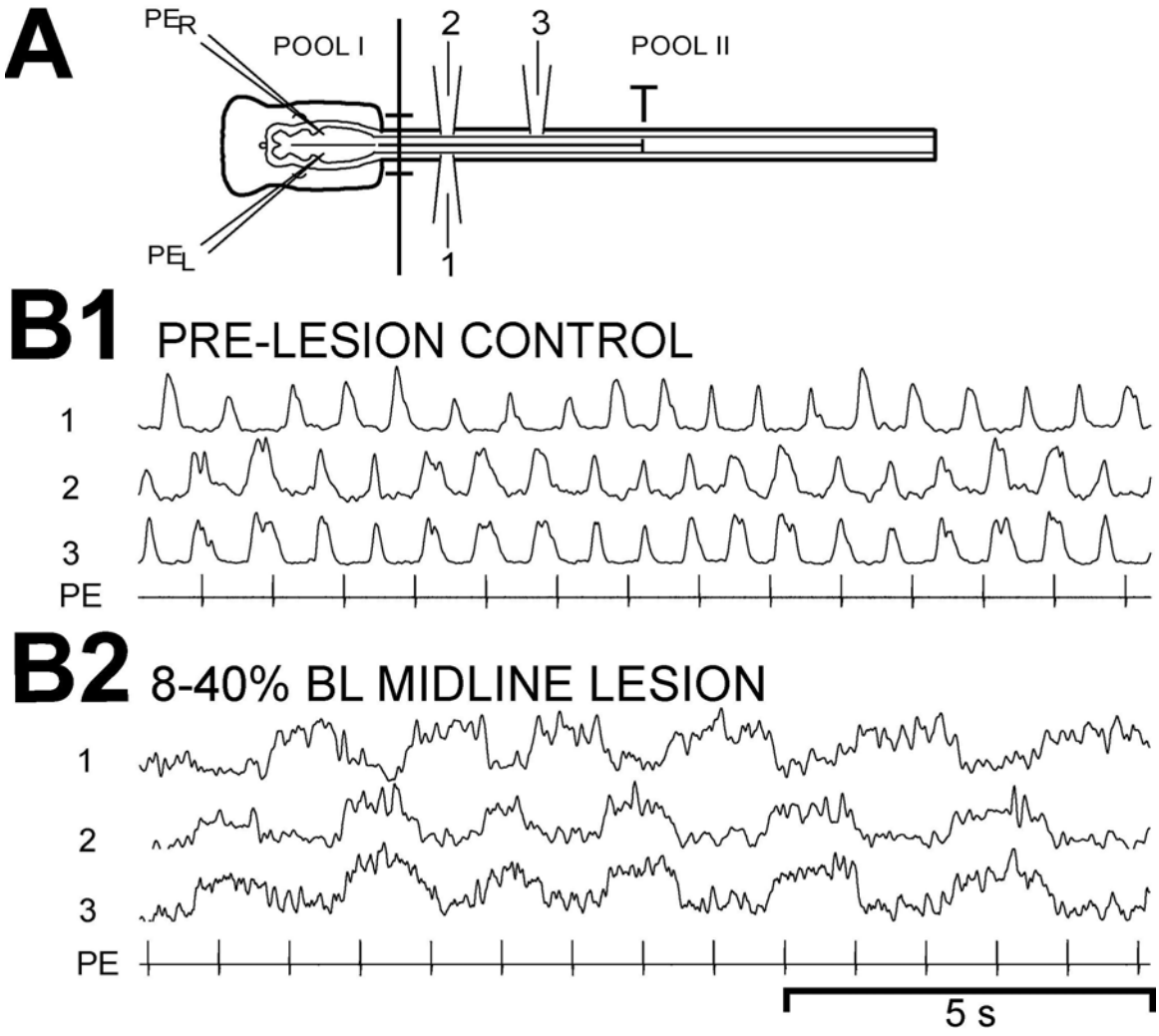
A
PER
POOL I
2
3
POOL II
T
PEL
1
B1
PRE-LESION CONTROL
1
2
3
PE
B2
8-40% BL MIDLINE LESION
1
2
3
PE
5 s

Figure 31

Figure 32. Origin of slow rhythmic burst activity in rostral hemi-spinal cords. (A) Partitioned i*n vitro* brain/spinal cord preparation (see Fig. 31) showing brain pool (I), spinal cord pool (II), pharmacological microstimulation pipettes, spinal cord fascicle electrodes (SC1, SC2), and midline lesion in the rostral spinal cord (horizontal line, 8%→40% BL; n = 4). (B1) Following a rostral midline lesion, stimulation in brain locomotor areas elicited slow rhythmic burst activity in ventral roots (not shown; see Fig. 31B2) and slow alternating burst activity in left and right spinal cord fascicles (SC1, SC2). (B3) Following blockade of synaptic transmission in the spinal cord (Pool II) with a low calcium Ringer's solution, brain stimulation still elicited slow alternating burst activity in spinal cord fascicles.

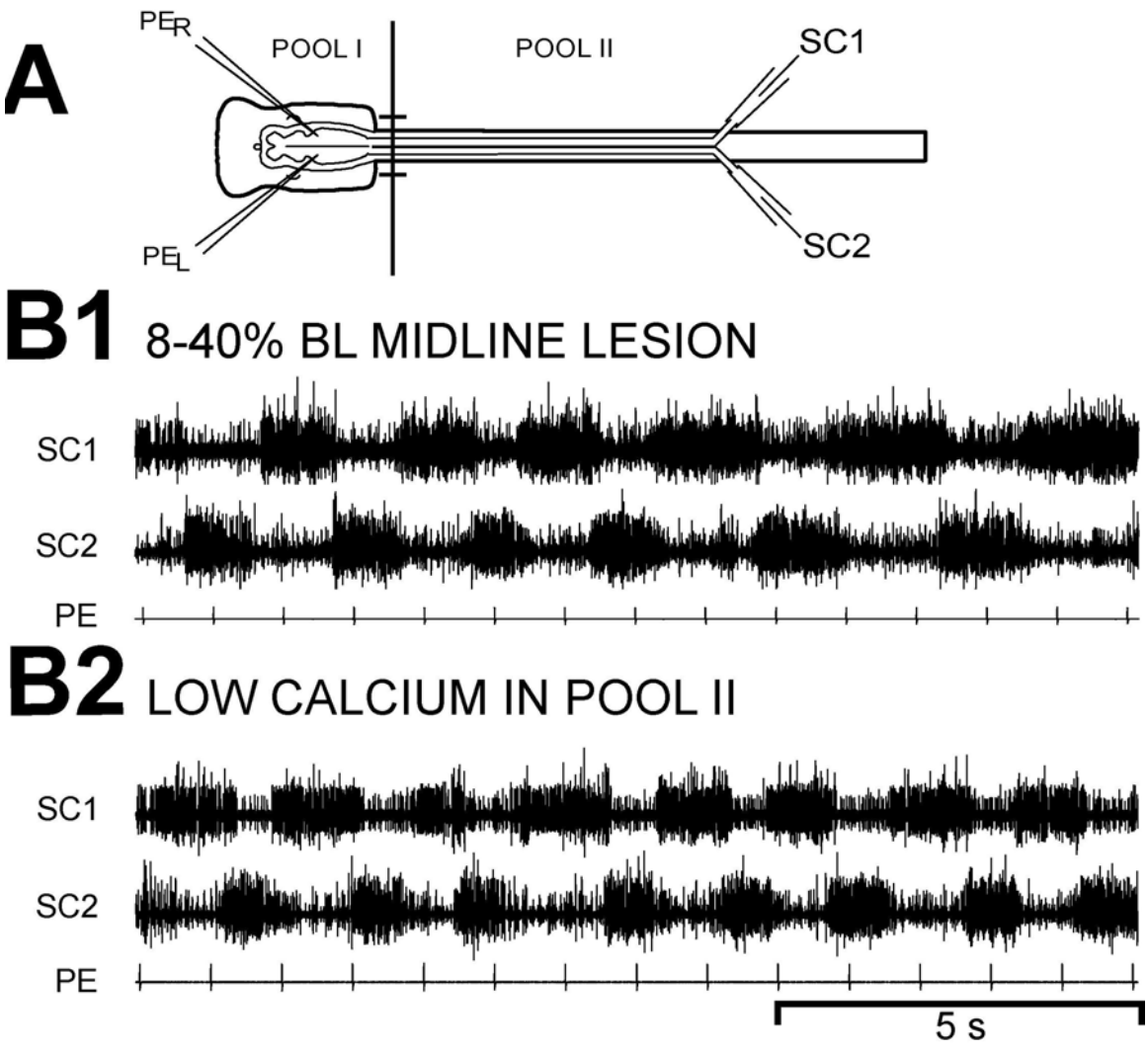
A
PER
POOL I
POOL II
SC1
SC2
PEL
B1 8-40% BL MIDLINE LESION
SC1
SC2
PE
B2 LOW CALCIUM IN POOL II
SC1
SC2
PE
5 s

Figure 32

Table 1

Normalized maximum lateral displacement versus normalized distances along the body for semi-intact preparations

Stim. Site		20 - 40% BL[a]	40 - 60% BL	60 - 80% BL	80 - 100% BL
RLR	RT	0.0175 ± 0.0151	0.0503 ± 0.0215	0.0736 ± 0.0246	0.1279 ± 0.0393
N = 7	LT	0.0146 ± 0.0129	0.0501 ± 0.0166	0.0699 ± 0.0220	0.1302 ± 0.0424
DLM	RT	0.0250 ± 0.0125	0.0641 ± 0.0151	0.0840 ± 0.0257	0.1724 ± 0.0562
N = 6	LT	0.0225 ± 0.0164	0.0711 ± 0.0158	0.0901 ± 0.0231	0.1766 ± 0.0552
VMD	RT	0.0206 ± 0.0164	0.0524 ± 0.0151	0.0684 ± 0.0244	0.1232 ± 0.0500
N = 7	LT	0.0136 ± 0.0126	0.0459 ± 0.0149	0.0632 ± 0.0232	0.1257 ± 0.0515

Stim. Site		20 - 40% BL	40 - 60% BL	60 - 80% BL	80 - 100% BL
aARRN	RT	0.0098 ± 0.0095	0.0487 ± 0.0174	0.0644 ± 0.0146	0.1029 ± 0.0316
N = 8	LT	0.0100 ± 0.0086	0.0435 ± 0.0163	0.0612 ± 0.0161	0.0991 ± 0.0345
pMRRN	RT	0.0147 ± 0.0125	0.0420 ± 0.0137	0.0609 ± 0.0257	0.1099 ± 0.0671
N = 6	LT	0.0125 ± 0.0110	0.0413 ± 0.0204	0.0623 ± 0.0335	0.1208 ± 0.0621
PRRN	RT	0.0100 ± 0.0105	0.0442 ± 0.0165	0.0605 ± 0.0185	0.1009 ± 0.0403
N = 8	LT	0.0097 ± 0.0103	0.0438 ± 0.0219	0.0591 ± 0.0221	0.1034 ± 0.0393

a – Since semi-intact preparations were immobile from ~0–20% BL (see Methods), no kinematic analysis was performed for the very rostral part of the preparation

Table 2

Kinematic parameters of locomotor movements

	T (ms)	WAVELENGTH λ	MECHANICAL ϕ LAG
WHOLE ANIMALS N = 6	215 ± 76	0.863 ± 0.112†	0.011 ± 0.0014
SEMI - INTACT			
RLR N = 7	429 ± 50	0.787 ± 0.066††††	0.011 ± 0.0012
DLM N = 6	924 ± 490***	0.840 ± 0.058††	0.011 ± 0.0007
VMD N = 7	448 ± 83	0.778 ± 0.101††	0.011 ± 0.0014
aARRN N = 8	446 ± 114	0.730 ± 0.148††	0.012 ± 0.0027
pMRRN N = 6	1133 ± 406***	0.645 ± 0.071††††	0.013 ± 0.0015
PRRN N = 8	745 ± 240**	0.750 ± 0.101†††	0.011 ± 0.0016

† – $P \leq 0.05$ (significantly different than $\lambda = 1.0$; Student's t-test); †† – $P \leq 0.002$; ††† – $P \leq 0.0002$; †††† – $P \leq 0.0001$

** – $P \leq 0.01$; *** – $P \leq 0.002$ (significantly different than corresponding parameter in whole animals; ANOVA)

Table 3

Parameters of locomotor muscle burst activity for each stimulation area

Stim. Site	T (ms)	BP_{ROST}	BP_{CAUD}	ϕ_{INT}	ϕ_{RT-LT}
Whole Animals [a]	342 ± 79 7/595 [b]	0.373 ± 0.075 7/595	0.361 ± 0.081 7595	0.006 ± 0.002 7/595	0.520 ± 0.100 10/367
RLR	686 ± 263 15/914	0.335 ± 0.106 15/914	0.255 ± 0.137 7/258	0.005 ± 0.004 7/258	0.487 ± 0.049 8/656
DLM	736 ± 377* 10/629	0.323 ± 0.107 10/629	0.289 ± 0.115 4/226	0.005 ± 0.003 4/226	0.494 ± 0.061 6/403
VMD	687 ± 290 14/764	0.307 ± 0.117 14/764	0.207 ± 0.096 6/257	0.004 ± 0.002 6/257	0.505 ± 0.054 8/507
aARRN	882 ± 305 2/114	0.311 ± 0.114 2/114	---	---	0.492 ± 0.079 2/114
pMRRN	2408 ± 933 1/32	0.434 ± 0.090 1/32	---	---	0.463 ± 0.061 1/32
PRRN	538 ± 233 3/185	0.385 ± 0.132 3/185	---	---	0.466 ± 0.072 3/185

a – Parameters for control locomotor muscle activity recorded in normal whole animals (Davis et al. 1993, Paggett et al. 1998).

b – n1/n2, where n1 = numbers of animals and n2 = numbers of cycles of rhythmic locomotor activity

* – $P \leq 0.05$ (significantly different than corresponding parameter in control animals; ANOVA)

Table 4

Parameters of Locomotor Activity initiated by Pharmacological Microstimulation with D-Glut/D-Asp in Different Brain Locomotor Areas[a]

Locomotor Area	T (ms)	BProstral	BPcaudal	Φ_{RT-LT} (rostral)	Φ_{INT}	LATENCY (sec)
WHOLE ANIMALS	342 ± 79 7/595[b]	0.373 ± 0.075 7/595	0.361 ± 0.081 7/595	0.520 ± 0.100 10/367	0.006 ± 0.002 7/595	
RLR (N=5)	1117 ± 351*	0.307 ± 0.065	0.252 ± 0.076	0.564 ± 0.076	0.002 ± 0.002	19 ± 7 (10-35)[c]
DLM (N=5	1470 ± 531**	0.269 ± 0.080	0.283 ± 0.116	0.490 ± 0.067	0.004 ± 0.003	12 ± 7 (6-29)
VMD (N=5)	1095 ± 313*	0.334 ± 0.063	0.326 ± 0.108	0.523 ± 0.099	0.003 ± 0.003	11 ± 4 (5-17)

a – parameters are for the "best site" in a particular brain locomotor area (see Methods)
b – number of animals/number of cycles
c – range of latencies for evoked locomotor activity

* - $P \leq 0.01$; ** - $P \leq 0.001$ - significantly different than cycle times for locomotor activity in whole animals (ANOVA)

Table 5

Parameters of Locomotor Activity initiated by Different Pharmacological Agents in Brain Locomotor Areas[a]

Locomotor Area	T (ms)	BProstral	BPcaudal	Φ_{RT-LT} (rostral)	Φ_{INT}	Latency (sec.)
RLR						
Glut/Asp (N = 8)	1051 ± 374	0.349 ± 0.069	0.333 ± 0.093	0.496 ± 0.078	0.005 ± 0.003	16 ± 9 (5-40)[c]
NMDA (N = 6)	1246 ± 476	0.388 ± 0.070	0.358 ± 0.107	0.520 ± 0.087	0.005 ± 0.003	30 ± 12 (16-50)
AMPA (N = 4)	1466 ± 572	0.313 ± 0.077	0.296 ± 0.128	0.484 ± 0.127	0.005 ± 0.003	13 ± 15 (2-45)
KA (N = 2)	1131 ± 246	0.335 ± 0.065	0.298 ± 0.060	0.529 ± 0.070	0.005 ± 0.002	5 ± 2 (3-7)
DLM (N = 3)						
Glut/Asp	893 ± 319	0.362 ± 0.053	0.304 ± 0.080	0.579 ± 0.080	0.002 ± 0.003	21 ± 14 (4-45)
NMDA	817 ± 256	0.362 ± 0.061	0.297 ± 0.060	0.617 ± 0.071	0.003 ± 0.003	32 ± 15 (11-52)
AMPA	907 ± 374	0.342 ± 0.057	0.292 ± 0.062	0.590 ± 0.074	0.002 ± 0.003	13 ± 18 (2-54)
KA	968 ± 359	0.350 ± 0.056	0.285 ± 0.062	0.581 ± 0.081	0.003 ± 0.003	21 ± 19 (2-51)
VMD (N = 3)						
Glut/Asp	958 ± 469	0.346 ± 0.115	0.353 ± 0.149	0.532 ± 0.179	0.003 ± 0.003	12 ± 4 (4-18)
NMDA	930 ± 191	0.337 ± 0.045	0.356 ± 0.069	0.495 ± 0.080	0.003 ± 0.002	27 ± 11 (11-44)
AMPA	1053 ± 367	0.348 ± 0.053	0.390 ± 0.101	0.458 ± 0.092	0.005 ± 0.003	11 ± 12 (3-35)
KA	957 ± 358	0.346 ± 0.056	0.304 ± 0.120	0.566 ± 0.087	0.005 ± 0.003	13 ± 9 (2-26)

a – parameters are for the "best site" in a particular brain locomotor area (see Methods)
b – number of animals/number of cycles
c – range of latencies for evoked locomotor activity

Table 6

Individual and Average Sizes of Higher Order Locomotor Areas[a]

Locomotor Area	Experiment Number	Width[b] (μm)	Length[c] (μm)
RLR (N = 5)	AJ 252	110	60
	AJ 250	65	190
	AJ 249	70	300
	AJ212	100	190
	AJ 211	375	400
	MEAN	144 ± 131	228 ± 128
VMD (N = 5)	AJ 239	92	376
	AJ 212	90	400
	AJ 210	60	125
	AJ 209	375	120
	AJ 208	50	50
	MEAN	133 ± 136	214 ± 162
DLM (N = 5)	AJ 240	420	100
	AJ 213	220	125
	AJ 212	150	200
	AJ 208	165	70
	AJ 207	265	110
	MEAN	244 ± 109	134 ± 49

a – pharmacological stimulation with 5 mM D-glutamate/D-aspartate
b – maximum extent along the transverse axis
c – maximum extent along the rostrocaudal axis

Note that brain locomotor areas are not square (see Figs. 3B,5B,7B), and the full area of a locomotor region was less than width * length

TABLE 7

RHYTHMIC MUSCLE ACTIVITY: WHOLE ANIMALS

Lesion Type	T	BP_{ros}	BP_{caud}	$\phi_{RT\text{-}LT\ (ROS)}$	$\phi_{RT\text{-}LT\ (CAUD)}$	ϕ_{INT}
Control Animals[a]	342 ± 79	0.373 ± 0.075	0.361 ± 0.081	0.520 ± 0.100	0.510 ± 0.080	0.0064 ± 0.0017
	7/595[b]	7/595	7/595	10/367	10/367	7/595
"Short" Caudal Longitudinal Lesion (35-45% BL)						
w/o T @ 45% BL (6/559[b])	239 ± 55	0.364 ± 0.061	0.479 ± 0.099	0.494 ± 0.058	0.470 ± 0.093	0.0077 ± 0.0025
w/ T @ 45% BL (6/497)	202 ± 39	0.365 ± 0.065	0.383 ± 0.109	0.502 ± 0.045	0.477 ± 0.076	0.0054 ± 0.0017
"Long" Caudal Longitudinal Lesion (30-50% BL)						
w/o T @ 50% BL (6/511)	216 ± 38	0.379 ± 0.053	0.487 ±0.083	0.507 ± 0.085	0.475 ± 0.098	0.0078 ± 0.0022
w/ T @ 50% BL (6/562)	187 ± 31	0.348 ± 0.060	0.463 ± 0.121	0.456 ± 0.109	0.357 ± 0.190	0.0068 ± 0.0022
Rostral Longitudinal Lesion (8-30% BL)						
w/o T @ 30% BL (7/457)[c]	319 ± 63	0.455 ± 0.130	0.433 ± 0.129	0.485 ± 0.117	0.500 ± 0.093	0.0002 ±0.0053*╪
w/o T @ 30% BL (3/83)[d]	551 ± 350	None	0.316 ± 0.099	None	0.490 ± 0.085	None
w/ T @ 30% BL (n=9)[e]	None	None	None	None	None	None

a - Control locomotor activity from normal animals from Davis et al. 1993 and Paggett et al. 1998
b – n1/n2, where n1 = numbers of animals and n2 = numbers of cycles of rhythmic motor activity
c - Animals in which muscle burst activity was present in both rostral and caudal body musculature (see Fig. 4B,5B1)
d - Animals in which muscle burst activity was only present in caudal body musculature
e - Animals in which recordings were made after spinal transections (n = 6), or before as well as after transections in the same animals (n = 3)
* $p < 0.01$ (significantly different than control locomotor activity; one-way ANOVA)
╪ $p > 0.05$ (not significantly different than 0; Student's t-test)

TABLE 8

RHYTHMIC VENTRAL ROOT ACTIVITY: IN VITRO BRAIN/SPINAL CORD PREPARATIONS

Lesion Type	T	BP_{ros}	BP_{caud}	$\phi_{RT\text{-}LT\ (ROS)}$	$\phi_{RT\text{-}LT\ (CAUD)}$	ϕ_{INT}
Caudal Longitudinal Lesion (30-50% BL)						
Pre-Lesion Control (13/1212[a])	919 ± 203	0.359 ± 0.069	0.368 ± 0.080	0.504 ± 0.089	0.485 ± 0.078	0.0032 ± 0.0016
w/o T @ 50% BL (13/474)	894 ± 493	0.416 ± 0.070	0.494 ± 0.113*	0.483 ± 0.127	0.467 ± 0.147	-0.0002 ± 0.0024**╪
w/ T @ 50% BL (7/339)	996 ± 338	0.430 ± 0.066	0.508 ± 0.093*	0.429 ± 0.156	0.449 ± 0.149	0.0002 ± 0.0018*╪
Rostral Longitudinal Lesion (8-30% BL)[b]						
Pre-Lesion Control (3/297)	833 ± 176	0.369 ± 0.047	0.392 ± 0.064	0.558 ± 0.080	0.552 ± 0.084	0.0033 ± 0.0015
w/o T @ 50% BL (3/131)	760 ± 193	None	0.350 ± 0.081	None	0.532 ± 0.072	None
w/ T @ 50% BL (n = 3/0)	None	None	None	None	None	None
Rostral Longitudinal Lesion (8% to 30 or 40% BL)[c]						
Pre-Lesion Control	961 ± 394 (7/541[a])	0.412 ± 0.066 (7/541)	0.425 ± 0.090 (7/541)	0.486 ± 0.090 (7/541)	0.485 ± 0.064 (3/250)	0.0036 ± 0.0028 (7/541)
w/o T @ 30% BL (3/104)	1461±1148	None	0.448 ± 0.088	None	0.488 ± 0.134	None
w/ T @ 30 or 40% BL[d]	3752±2028 (7/241)	0.663 ± 0.083*** (7/241)	0.643 ± 0.113* (4/132)	0.482 ± 0.104 (n=7/241)	None	0.0006 ± 0.0019***† (4/117)

a – n1/n2, where n1 = numbers of animals and n2 = numbers of cycles of rhythmic motor activity

b – following rostral midline lesions and spinal cord transections at 30% BL, alternating burst activity was not present in rostral ventral roots (see Fig. 6B3)

c – following rostral midline lesions and spinal cord transections at 30% or 40% BL, slow alternating burst activity was present in rostral ventral roots (see Fig. 7B3)

d – rostral burst activity was recorded at ~20% BL (n = 3; T @ 30% BL; see Fig. 7A), or rostral and caudal burst activity were recorded at ~20% and ~35% BL, respectively (n = 4; T @ 40% BL; see Fig. 8A)

* - $p < 0.01$; ** - $p < 0.001$ (significantly different than pre-lesion control locomotor activity; one-way ANOVA)

*** - $p < 0.001$ (significantly different than pre-lesion control locomotor activity; Student's t-test)

† $p < 0.001$ (significantly different than 0; Student's t-test)

╪ $p > 0.05$ (not significantly different than 0; Student's t-test)

DISCUSSION

Movements and Muscle Activity Initiated by Stimulation in Higher-Order Locomotor Areas (Ch. III)

1. *Brief Summary of Results*

In the present study in semi-intact preparations from larval lamprey, pharmacological microstimulation was applied to higher order locomotor areas or reticular nuclei. First, bilateral pharmacological microstimulation in higher locomotor areas (VMD, RLR or DLM) initiated symmetrical swimming movements and well-coordinated locomotor muscle activity. In contrast, unilateral stimulation in the above higher order locomotor areas elicited asymmetrical undulatory movements, most of which appeared to represent smooth, asymmetrical swimming. In particular, unilateral stimulation in VMD/DLM (RLR), for the most part, produced movements that were skewed toward (away from) the side of stimulation. These asymmetrical responses may be an indication of the symmetry with which these locomotor areas connect with neural elements "downstream" in the command pathway or the nature of interconnections between both sides of the brain. Finally, stimulation in brain regions just outside of the above higher locomotor areas was ineffective or elicited spastic flexion movements. Thus, pharmacological stimulation appeared to be relatively focal, suggesting that the concentration of agents decreased sharply with increasing distance from the micropipette tips (Curtis, 1964).

Second, with synaptic transmission blocked in the brain, bilateral pharmacological microstimulation in certain regions in reticular nuclei (aARRN,

pMRRN, or all of the PRRN) initiated symmetrical swimming movements and well-coordinated locomotor muscle activity. Bilateral stimulation in the pARRN, which contains relatively few descending brain neurons (Davis and McClellan, 1994a), usually did not elicit movements or muscle activity. In contrast, stimulation in the aMRRN, which contains several larger Müller cells (Rovainen, 1978), usually produced pronounced flexure responses or writhing. Unilateral pharmacological microstimulation in reticular nuclei (aARRN, pMRRN, or PRRN) elicited asymmetrical swimming-like movements or poorly coordinated rhythmic movements that did not resemble swimming. Interestingly, in *in vitro* brain/spinal cord preparations, pharmacological microstimulation in reticular nuclei does not reliably initiate spinal locomotor activity (Hagevik et al., 1996). This might be due, in part, to the presumed higher degree of excitability in the nervous systems of semi-intact preparations compared to *in vitro* preparations.

In semi-intact preparations, the cycle times for swimming activity initiated from the DLM, pMRRN, or PRRN typically were longer than but overlapped with those for swimming in whole animals. This is perhaps not surprising, since in *in vitro* brain/spinal cord preparations from larval lamprey, cycle times of locomotor activity tend to be longer than those during swimming in whole animals (Davis et al., 1993; McClellan, 1994). Differences in cycle times of locomotor activity in whole animals and reduced preparations (e.g. semi-intact or *in vitro* brain/spinal cord preparations) probably are due, in part, to differences in central nervous system excitability or sensory inputs (also see Calabrese and Kristan, 1976; Yakovenko et al., 2005).

Aside from cycle times, the parameters of locomotor burst activity initiated from higher order locomotor areas or reticular nuclei were not significantly different than those

for swimming in whole animals. In contrast, in *in vitro* brain/spinal cord preparations, pharmacological stimulation in the RLR, DLM, or VMD initiates spinal locomotor activity in which the intersegmental phase lags are significantly smaller than those during swimming in whole animals. This result does not appear to be due to the nature of pharmacological microstimulation in brain locomotor areas. For example, in semi-intact preparations, pharmacological microstimulation in these same higher order locomotor areas initiates locomotor muscle activity with intersegmental phase lags that are not significantly different than those during swimming in whole animals (Table 3). Thus, in larval lamprey, sensory feedback may contribute to the generation of proper phase lags of spinal locomotor activity (Hagevik and McClellan, 1994; McClellan, 1994), perhaps due to immature spinal circuitry or to a lack of or immaturity of some cell types in the spinal CPGs compared to those in adults (Cohen et al., 1990).

In adult lamprey, the parameters of locomotor activity generated by whole animals swimming in a "swim mill" are not significantly different than those for swimming activity produced by spinalized animals or those during fictive locomotion initiated by bath application of D-glutamate in isolated spinal cord preparations (Wallen and Williams, 1984). Thus, in contrast to larval lamprey, the spinal CPGs in adult animals can generate the basic pattern of locomotor activity, including proper intersegmental phase lags, in the absence of sensory feedback.

2. *Organization of Brain Locomotor Areas*

Neuronal blocking experiments in previous studies suggest that neurons in the RLR locomotor areas project to the DLM and VMD, which then in turn project to reticular nuclei (Paggett et al., 2004). This model is supported by additional experiments

in which lesions at the mesencephalon-rhombencephalon border abolish RLR-initiated locomotion, suggesting that at least some neurons in the RLR project rostrally (Paggett et al., 2000). Data from the present study indicate that all of these brain locomotor areas can initiate well-coordinated locomotion and swimming activity. Preliminary mapping studies indicate that the RLR, DLM, and VMD locomotor areas are restricted in size, and that all three ionotropic receptors for excitatory amino acids are present (Jackson et al., 2006).

3. *Comparison to Other Lamprey Studies*

In previous studies in adult lamprey, a region in the ventral thalamus, the diencephalic locomotor region (DLR), has been partially characterized both anatomically and functionally (El Manira et al., 1997; Menard et al., 2005). The DLR in adults appears to be very similar in location to the VMD in larval lamprey that was functionally characterized in the present and previous studies (Paggett et al., 2004). First, stimulation in this brain area in *in vitro* brain/spinal cord preparations initiates spinal locomotor activity and elicits monosynaptic responses in RS cells (El Manira et al., 1997; Paggett et al., 2004). Furthermore, application of retrograde tracer to reticular nuclei labels neurons in the ventral thalamus in adult (El Manira et al., 1997) and larval (Paggett, 1999) lamprey. In adult lamprey, the DLR receives projections from several different regions in the brain and may control the level of excitability in RS neurons, which then activate CPGs in the spinal cord (El Manira et al., 1997). In semi-intact preparations from adult lamprey, electrical or pharmacological stimulation in the DLR appears to produce symmetrical swimming movements (Menard et al., 2005). Also, the frequency (i.e. power) of swimming apparently was not graded with variations in intensity or frequency

of electrical stimulation (Menard et al, 2005). However, in this study, it is unclear whether bilateral or unilateral stimulation was used, how the symmetry of swimming movements was assessed, or if muscle recordings were performed. In the present study in larval animals, bilateral pharmacological microstimulation in the VMD initiated symmetrical swimming movements, and the parameters of muscle burst activity were similar to those during swimming in whole animals.

Unilateral electrical or pharmacological microstimulation in the MLR elicits symmetrical swimming in semi-intact preparations from both larval and adult lamprey, and the frequency (i.e. power) of swimming could be graded by varying the intensity of electrical stimulation (Sirota et al., 2000; also see McClellan and Grillner, 1984). Similar to the VMD (or DLR), electrical stimulation in the MLR elicits monosynaptic responses in RS neurons, and application of a retrograde tracer to the MRRN labels neurons in the MLR (Sirota et al., 2000; also see McClellan, 1989). The exact location of the MLR in larval lamprey is difficult to ascertain since the published anatomical diagrams are from adults. However, all available evidence strongly suggests that the DLM (Paggett et al., 2004; present study) and MLR (Sirota et al., 2000) are separate brain locomotor areas. In contrast to unilateral activation of the MLR, unilateral stimulation in the VMD, DLM, or RLR usually elicits asymmetrical undulatory movements. However, the symmetry of movements initiated by these brain locomotor areas may be largely an indication of connectivity to "downstream" targets. In particular, brain locomotor areas undoubtedly are bilaterally active during normal initiation of swimming, and so bilateral stimulation is a more physiologically realistic test of the function of these brain areas.

In lamprey, RS neurons are the neural output elements of the locomotor command system that directly activates spinal CPGs and initiates locomotor activity (McClellan, 1988; Shaw et al., 2001; see Brodin et al., 1988). Interestingly, in semi-intact lamprey preparations in which synaptic transmission was not blocked in the brain, unilateral electrical stimulation in the MRRN and ARRN occasionally elicited only a few cycles of swimming that quickly deteriorated into tonic contractions, while similar stimulation in the PRRN elicited spastic muscle contractions (Sirota et al., 2000). In contrast, in the present study with semi-intact preparations from larval lamprey, chemical synaptic transmission was blocked in the brain to ensure that motor responses evoked by pharmacological stimulation were due to RS neurons. Under these conditions, bilateral pharmacological stimulation in the PRRN as well as parts of the ARRN and MRRN initiated well-coordinated, symmetrical swimming. There are several possible explanations for the differences in these two studies. First, as stated above, bilateral stimulation in brain locomotor areas is a more physiological test of function than unilateral stimulation. For example, in the present study, unilateral pharmacological stimulation in reticular nuclei elicited asymmetrical undulations or flexure movements. Second, in addition to activation of RS neurons, electrical stimulation in reticular nuclei may have activated axons in the MLF, axons of passage, and input axons that terminated in reticular nuclei. These axons undoubtedly have different functions, and thus, stimulation in reticular nuclei may have elicited conflicting responses. Third, electrical stimulation in reticular nuclei might have activated fewer RS neurons than pharmacological stimulation in these brain areas.

In other studies, unilateral electrical stimulation in the MLR produced larger synaptic responses in RS neurons in the MRRN than the PRRN, suggesting that RS neurons in the MRRN are active during low frequency swimming while neurons in the PRRN are recruited for higher frequency swimming (Brocard and Dubuc, 2003). However, there are several issues to consider. First, during normal locomotion, several brain locomotor areas may be active, possibly including the MLR, and the recruitment order of RS neurons under these conditions is unknown. Second, RS neurons in the aARRN also can initiate swimming, and the recruitment order of these RS neurons must also be taken into consideration. In addition, pharmacological stimulation in the aARRN or PRRN initiated locomotor movements with significantly shorter cycle times than stimulation in the pMRRN. Third, it cannot be excluded that relatively large numbers of PRRN neurons with low levels of activity are more effective than relatively few, highly active MRRN neurons. For example, the strength with which individual RS neurons in different nuclei activate spinal CPGs has not been investigated in detail.

4. *Studies in Other Animals*

Surprisingly, relatively few studies have compared brain-initiated "fictive" locomotor patterns with the motor patterns that result from descending activation in semi-intact or whole animal preparations. In decerebrate cats, electrical stimulation in the MLR produces well-coordinated walking on a treadmill (Shik et al., 1966), and the pattern of muscle burst activity is similar to that during walking in intact cats (Grillner and Zangger, 1984). Following a complete transection of the dorsal roots, the basic pattern of muscle burst activity elicited by MLR stimulation is retained (Grillner and Zangger, 1984). In paralyzed, decerebrate cats, unilateral electrical microstimulation in

the MLR elicits a pattern of ventral root burst activity, termed "fictive" locomotion, that displays the same basic properties as MLR-initiated locomotor activity in animals walking on a treadmill (Jordan et al., 1979; Amemiya and Yamaguchi, 1984; Bem et al., 1993; also see Fetcho and Svoboda, 1993). Possible differences between "fictive" locomotor patterns and motor patterns in intact animals may be due, in part, to mechanosensory inputs to spinal CPG.

5. *Conclusions*

In the present study using semi-intact preparations from larval lamprey, bilateral pharmacological microstimulation in higher order brain locomotor areas (RLR, VMD, or DLM) initiated symmetrical swimming movements and well-coordinated muscle burst activity that were very similar to those during free swimming in whole animals. Likewise, bilateral stimulation in several regions in reticular nuclei also initiated symmetrical swimming movements and locomotor muscle activity. In contrast, stimulation outside of these areas did not initiate swimming movements. Importantly, unilateral stimulation in any of the above areas usually elicited asymmetrical movements. In conclusion, the present study strongly suggests that ventral root activity initiated from the above brain locomotor areas in *in vitro* preparations underlies locomotion. In addition, in many studies unilateral stimulation has been used to locate and define brain locomotor areas, but results from the present study indicate that bilateral stimulation is a more physiologically realistic test of the function of these brain areas. The present study is one of the few to correlate brain-initiated *in vitro* spinal locomotor activity ("fictive" locomotion") with locomotion and locomotor activity in whole animals and semi-intact preparations.

Size and Pharmacology of Higher-Order Locomotor Command Areas (Ch. IV)

1. *Brief Summary of Results*

Before summarizing the results from the present study, several issues with regard to the use of pharmacological microstimulation for mapping brain locomotor areas should be discussed. First, pharmacological microstimulation is thought to activate cell bodies and dendrites, but not, as a rule, axons of passage (Goodchild et al., 1982). Second, pharmacological microstimulation appears to activate neural structures that are relatively close to the tips of stimulating micropipettes. For example, movement of the stimulating micropipettes by as little as 50 μm in the brain could result in dramatically different evoked activity (see Fig. 16C2 and 16C4). In addition, during intracellular recording from RS neurons, pressure ejection with 5 mM D-glutamate/D-aspartate could be as close as 75-100 μm to the soma before depolarizing potentials were elicited (unpublished observations). Third, pharmacological microstimulation probably did not activated dendrites of neurons that were located large distances from the immediate area of stimulation. As stated above, relatively small differences in the locations of pharmacological microstimulation could result in very different evoked spinal motor activity (see Fig. 16, 18, 20). Thus, the neural structures that were activated by pharmacological microstimulation appear to be confined to discrete regions of the brain (see Table 6).

In the present study in *in vitro* brain/spinal cord preparations from larval lamprey, pharmacological microstimulation in higher locomotor areas (VMD, DLM, or RLR; Hagevik and McClellan, 1994b; McClellan, 1994; McClellan and Hagevik, 1997; Paggett et al., 2004) was used to determine the sizes, pharmacology, and organization of the locomotor command system. First, bilateral stimulation in the DLM, VMD, or RLR locomotor areas initiated well-coordinated spinal locomotor activity, and mapping in and around the site indicated that the locomotor areas were restricted to small areas of the brain. The results suggest that these brain locomotor areas are discrete, specialized regions of the brain, and initiation of spinal locomotor activity was not due to activation of nonspecific neural elements. However, the sizes of effective brain locomotor areas were not constant and tended to be slightly larger in preparations that initiated locomotor activity with the shortest cycle times (Table 6). Interestingly, in decerebrate cats, the relative size of the MLR that can evoke forelimb locomotor movements depended upon whether the forelimbs were deafferented (fictive locomotion) or walking on a treadmill (Amemiya and Yamaguchi, 1984). Thus, the effective sizes of brain locomotor areas appear to depend, in part, on the general excitability of the nervous system. For example, in a highly excitable preparation neurons near the border of a given brain locomotor area could be activated above threshold more easily than in preparations with lower excitability. Finally, in semi-intact preparations from larval lamprey, bilateral pharmacological microstimulation with 5 mM D-glutamate/D-aspartate in the VMD, DLM, or RLR elicits locomotor movements and locomotor muscle activity that are similar to those during swimming in whole animals (Jackson and McClellan, 2001; Jackson et al., 2006).

Second, pharmacological microstimulation in the DLM and VMD with NMDA, AMPA, or kainate initiated well-coordinated spinal locomotor activity, suggesting that all three ionotropic EAA receptors are present in these locomotor areas and contribute to brain-initiated spinal locomotor activity. Stimulation in the RLR with NMDA and AMPA elicited well-coordinated spinal locomotor activity in most preparations, while stimulation with kainate did not reliably initiate spinal locomotor activity. These differences with which application of different pharmacological agents to the RLR initiated spinal locomotor activity may be due to differences in the density of EAA receptors. Although all three EAA receptor subtypes are present in the above locomotor areas, it is possible that other neurotransmitters and their receptors (e.g. acetylcholine) could contribute to the initiation of locomotion.

2. *Organization of Brain Locomotor Areas*

In the present study, with synaptic transmission blocked in the brain, pharmacological microstimulation in the DLM, VMD, or RLR no longer initiated spinal locomotor activity, suggesting that neurons in these areas do not directly activate spinal locomotor networks. The DLM and VMD locomotor areas appear to make direct connections with RS neurons, since focally blocking neural activity in reticular nuclei abolishes or attenuates DLM- or VMD-initiated spinal locomotor activity (Paggett et al., 2004). Also, brief electrical stimulation in the DLM or VMD elicits monosynaptic responses in RS neurons, and DLM- and VMD- evoked synaptic responses summate in RS neurons (Paggett et al., 2004; also see El Manira et al., 1997). Application of retograde tracer to reticular nuclei labels neurons in the vicinity of the DLM and VMD (Paggett et al., 2001; El Manira et al., 1997). Finally, in semi-intact preparations with

synaptic transmission blocked in the brain, stimulation in reticular nuclei can still initiate spinal locomotor activity (Jackson and McClellan, 2001; Jackson et al., 2006).

Furthermore, in the present study, following a complete transection at the mesencephalic-rhombencephalic border, stimulation in the RLR no longer initiated spinal locomotor activity, suggesting that more rostral structures (e.g. DLM, VMD) may be required for RLR-initiated locomotion. Also, in whole animals, a transection at the mesencephalic-rhombencephalic border blocks initiation of locomotor behavior (McClellan, 1988). In support of this notion, focal blockade of neuronal activity in the DLM or VMD abolishes or greatly attenuates RLR-initiated spinal locomotor activity. Also, application of retrograde tracer in DLM or VMD labels neurons in the vicinity of the RLR locomotor area (Pagget et al., 2001). These results and the experiments in the present study suggest that neurons in the RLR locomotor areas project rostrally to the DLM and VMD, which then in turn project caudally to and activate RS neurons in reticular nuclei (see Fig. 33) (Paggett et al., 2004).

RLR → VMD and DLM → RS neurons → spinal locomotor networks

This model certainly does not include all aspects of the command system for swimming. For example, other sensory modalities (e.g. vision, olfaction) are not included but appear to have inputs to the command system, and other brain locomotor areas (e.g. MLR) have been omitted because the inputs to these regions have not be determined.

Preliminary results suggest that the RLR may receive sensory inputs from the trigeminal system, which transmits sensory information from the oral hood and head. First, sensory stimulation of the oral hood can elicit escape swimming behavior (McClellan, 1984), suggesting that trigeminal sensory inputs project to the locomotor

command system. Second, afferents in the descending trigeminal tracts (dV, Fig. 4B) synapse with second order sensory neurons in the nucleus of the descending trigeminal tract (Northcutt, 1979) in the lateral rhombencephalon. Preliminary results indicate that injection of retrograde tracer in the RLR labels neurons in the lateral rhombencephalon in the general vicinity of the dV (Paggett, 1999).

Brief activation of trigeminal inputs can result in sustained bouts of swimming (McClellan, 1984), but the mechanisms for translating a brief input into a sustained response are not clear. One possibility is that RS neurons exhibit plateau potentials in response to trigeminal sensory inputs (Viana Di Prisco et al., 1997). However, if such a mechanism does contribute, it would seem more appropriate for it to reside in higher order areas of the locomotor command system that have inputs to and regulate the activity in RS neurons. Alternatively, there may be reverberatory circuits in the command system, as has been found in Xenopus (Li et al., 2006).

3. *Comparison to Other Studies in the Lamprey*

Second order trigeminal sensory neurons in the nucleus of the descending trigeminal tract (Northcutt, 1979) do make synapses with RS neurons (Viana Di Prisco et al., 2005), and it has been proposed that this disynaptic pathway is the mechanism by which trigeminal inputs initiate locomotion (Viana Di Prisco et al., 1997,2005; LeRay et al., 2004). In contrast, other evidence discussed above suggests that neural centers in the mesencephalon and perhaps the diencephalon are required for trigeminal evoked locomotor behavior (McClellan, 1988; Paggett et al., 2004). In addition, trigeminal-evoked locomotor behavior sometimes occurs after a delay and once initiated, often involves relatively long duration bouts of swimming and substantial exploration of the

environment. These features of sensory-evoked locomotion would seem to be too complex to be mediated by a relatively simple disynaptic reflex pathway, suggesting that more rostral higher order locomotor areas are involved in initiation of trigeminal-evoked locomotor behavior.

Neurons in higher order brain locomotor areas, such as the MLR, project to reticular nuclei and elicit monosynaptic responses in RS neurons (McClellan, 1989; Brocard and Dubuc, 2003). In *in vitro* brain/spinal cord preparations, electrical stimulation in this area initiates spinal locomotor activity (McClellan and Grillner, 1984), while in semi-intact preparations, stimulation initiates swimming movements and muscle burst activity (Sirota et al., 2000). During electrical stimulation in the MLR, increasing the intensity of stimulation increased the frequency of swimming activity (McClellan and Grillner, 1984; Sirota et al., 2000).

Electrical stimulation in the ventral thalamus, in an area referred to the "diencephalic locomotor region" (DLR), elicits monosynaptic responses in RS neurons (El Manira et al., 1997) initiates spinal locomotor activity (El Manira et al., 1997). Injection of retrograde tracer in reticular nuclei labels neurons in the vicinity of the DLR. Injection of tracer in the DLR labeled anatomical projections to all reticular nuclei, but not direct projections were found to the spinal cord (El Manira et al., 1997), in agreement with the physiological results in the present study (Fig. 22). The DLR in adults very likely is analogous in larval lamprey to the VMD, which also elicits monosynaptic responses in RS neurons, initiates *in vitro* spinal locomotor activity (Paggett et al, 2004), and contains labeled neurons after tracer injection in reticular nuclei (Paggett et al., 2001). Furthermore, in semi-intact preparations from larval lamprey, stimulation in the

VMD initiates well-coordinated swimming movements and muscle burst activity that are similar to those during swimming in whole animals (Jackson and McClellan, 2001; Jackson et al., 2006).

4. *Comparison to Brain Locomotor Areas in Other Vertebrates*

The general organization of brain locomotor areas in the lamprey appears to be similar to that found in "higher" vertebrates. First, in cat, activation of trigeminal afferents (Aoki and Mori, 1981) or stimulation in lateral areas of the brain, such as the "pontomedullary locomotor strip" (PLS), initiate locomotor activity (Mori et al., 1977; Kazennikov et al., 1981; Selionov and Shik, 1984; Steeves et al., 1987; Beresovskii and Bayev, 1988; Noga et al., 1988,1991; Sholomenko et al., 1991a,b). The PLS corresponds to the spinal nucleus of the trigeminal nerve (Bayev et al., 1988; Beresovskii and Bayev, 1988; Noga et al., 1988,1991; also see Garcia-Rill et al., 1983), which is analogous to the nucleus of the descending trigeminal tract in lamprey (Northcutt, 1979). Second, in mammals, stimulation in a subthalamic locomotor region (SLR) can produce synaptic responses in RS neurons (Orlovsky, 1970) and evoke locomotion (Orlovsky, 1969; Parker and Sinnamon, 1983; Skinner and Garcia-Rill, 1984). Third, the MLR initiates spinal locomotor activity in a wide variety of vertebrates (Shik et al., 1966; Kashin et al., 1974, 1981; Eidelberg et al., 1981; Parker and Sinnamon, 1983; Sinnamon, 1984; Grillner and Wallen, 1984; McClellan and Grillner, 1984; Amemiya and Yamaguchi, 1984; Garcia-Rill and Skinner, 1987; Milner and Mogenson, 1988; Coles et al., 1989; Bernau et al., 1991; Douglas et al., 1993; Fetcho and Svoboda, 1993; Uematsu and Todo, 1997; Sirota et al., 2000; Cabelguen et al., 2003; also see Parker and Sinnamon, 1983). Results from several studies suggest that the MLR does not directly activate the spinal

locomotor networks but instead activates RS neurons (Orlovsky, 1970; Steeves and Jordan, 1984; Garcia-Rill et al., 1986; Garcia-Rill and Skinner, 1987a,b; Skinner et al., 1990; reviewed in Jordan, 1986). Finally, RS neurons in the medioventral medulla are thought to activate spinal locomotor networks (Steeves et al., 1987; Noga et al., 1988; Atsuta et al., 1990; Livingston and Leonard, 1990; Kinjo et al., 1990; reviewed in Jordan, 1986). In particular, lesions or injection of pharmacological blockers in these nuclei can abolish MLR-elicited locomotor activity (Garcia-Rill and Skinner, 1987a; Bernau et al., 1991; Noga et al., 1991), and focal cooling in the medial reticular formation could block PLS-initiated locomotor activity (Noga et al., 1991).

5. *Conclusions*

In *in vitro* brain/spinal cord preparations from larval lamprey, pharmacological microstimulation in the DLM, VMD, or RLR initiated well-coordinated spinal locomotor activity. Mapping experiments indicated that like brain locomotor areas in mammals, those in the lamprey are restricted to discrete areas of the brain. However, it is likely that the effective size of locomotor command areas could vary depending on the overall excitability in different preparations.

Disruption of Left-Right Reciprocal Coupling in the Spinal Cord (Ch. V)

1. *Brief Summary of Results*

In the present study in both whole animals and *in vitro* brain/spinal cord preparations from larval lamprey, longitudinal midline lesions were made in the rostral (8-30% BL) or caudal (30-50% BL) spinal cord, and spinal motor activity was initiated from the brain. The results suggest that in the absence of connections with intact regions of cord, isolated left and right hemi-spinal cords are not able to generate locomotor burst

activity in response to descending activation from locomotor command systems in the brain. First, following midline lesions in the caudal spinal cords of both whole animals and *in vitro* preparations, left-right alternating burst activity was present in rostral and usually caudal parts of the body and spinal cord, respectively (Figs. 24,25). In *in vitro* preparations, blocking synaptic transmission in the rostral cord abolished locomotor-like burst activity in left and right caudal hemi-spinal cords (Fig. 26). Second, following midline lesions in the rostral spinal cords of whole animals, alternating muscle burst activity was present in the caudal and sometimes the rostral body (Figs. 27,28). However, following a spinal cord transection at 30% BL, locomotor-like burst activity originating from left and right rostral hemi-spinal cords was abolished. In contrast, for *in vitro* preparations with similar rostral midline spinal lesions, left-right alternating burst activity was produced in the caudal, intact spinal cord but not the rostral, lesioned cord (Fig. 29,30). Following a transection at 30% BL, very slow rhythmic burst activity was sometimes present in rostral hemi-spinal cords (Figs. 30,31), but the parameters of this activity were significantly different than those for locomotor activity in normal animals (Table 8). In addition, the rhythmicity for this slow rhythmic burst activity appeared to originate from descending neurons in the brain that project to the spinal cord (Fig. 32). Since large reticulospinal Müller cells have descending axons in the medial part of the cord, rostral midline spinal cord lesions might have irritated the axons of some of these neurons and lowered their thresholds. Since these descending brain neurons make chemical and electrical synapses with spinal motoneurons (reviewed in Rovainen, 1979), rhythmic activity in Müller cells during pharmacological microstimulation in brain

locomotor areas might have contributed to rhythmic activity in ventral roots (Fig. 30B3,31B2) and spinal cord fascicles (Fig. 32).

In the present study, it is unlikely that the absence of locomotor burst activity in isolated hemi-spinal cords (Figs. 26B2,27C,28B2,29B2,29B3) was due to excessive injury of spinal CPG modules. For example, in whole animals with midline lesions of the rostral spinal cord (8-30% BL), left-right alternating muscle burst activity sometimes was present in rostral musculature (Fig. 28B1) but was abolished in the same animals following a spinal transection at 30% BL (Fig. 28B2). Likewise, in *in vitro* preparations with midline lesions in the caudal spinal cord, left-right alternating burst activity usually was present in the caudal hemi-spinal cords (Fig. 26B1) but was abolished in the same animals following blockade of synaptic transmission in the rostral cord (Fig. 26B2).

Previous results suggest that right and left spinal CPG modules in larval lamprey are connected by relatively strong reciprocal inhibition in parallel with weaker reciprocal excitation (Hagevik and McClellan, 1994). For example, in *in vitro* brain/spinal cord preparations, application of strychnine to the spinal cord to block reciprocal inhibition converts brain-evoked left-right alternating burst activity to synchronous bursting. Although these results suggest that reciprocal inhibition is not required for rhythmogenesis, they do not prove that left and right CPG modules can function autonomously because these modules also are connected by reciprocal excitation. Unfortunately, it probably is not possible to selectively block both reciprocal excitation and inhibition with pharmacological agents without compromising the functions of the CPG modules themselves.

Taken together, the results suggest that in larval lamprey under the present experimental conditions, isolated left and right hemi-spinal cords are not capable of generating locomotor burst activity in response to descending activation from locomotor command systems in the brain. In addition, the results imply that reciprocal connections, mediate by commissural interneurons, between left and right spinal CPG modules contribute to both left-right phasing and rhythmogenesis of locomotor activity.

2. *Comparison to Other Studies in the Lamprey and Xenopus*

Two previous studies in which midline lesions were made in the spinal cords of adult lamprey appeared to obtain contradictory results. In one study, midline lesions spanned about half the length of *in vitro* spinal cord preparations, and rhythmic burst activity was elicited by bath application of NMDA (Buchanan, 1999). There was a rapid deterioration of left-right alternating burst activity with increasing distance along the lesioned section of spinal cord, while alternating burst activity was preserved in the unlesioned part of the spinal cord. These results were interpreted to mean that reciprocal inhibition in spinal CPGs, which appears to be mediated, in part, by crossed,contralateral descending interneurons (CCI's), contributes to rhythmogenesis. However, left and right spinal CPG modules are connected by relatively strong reciprocal inhibition in parallel with weaker reciprocal excitation (Hagevik and McClellan, 1994). Therefore, since midline lesions abolish both types of reciprocal connections, this type of lesion experiment does not, by itself, determine whether reciprocal inhibition or excitation is critical for rhythmogenesis.

In a second study, in which midline lesions spanned the entire length of *in vitro* spinal cord preparations from adult lamprey, rhythmic burst activity was elicited either by

bath application of pharmacological agents (i.e. NMDA or D-glutamate) or by brief electrical stimulation of the dorsal surface of the end of a hemi-cord (Cangiano and Grillner, 2003; also see Grillner et al., 1986; Kotaleski et al., 1999). In particular, electrical stimulation elicited two types of rhythmic ventral root burst activity: (a) "slow" rhythm with cycle times of ~2.5-10.0 s (mean ~5 s); and (b) "fast" rhythm with cycle times of ~0.08-0.5 s (mean ~0.2 s). The slow rhythm was not considered by the authors to correspond to swimming behavior. In contrast, the fast rhythm was thought to represent swimming motor activity, since progressively more complete midline spinal cord lesions resulted in a gradual transition from "normal" *in vitro* swimming activity to the "fast" rhythm.

There are several points to consider regarding the interpretations from the second study above. First, many of the cycle times of the "fast" rhythm are much shorter than those for swimming in normal adult lamprey (0.3 – 1.4 s, McClellan, 1984). Second, since burst proportions and rostrocaudal phase lags for the "fast" rhythm have not been reported, it is unclear whether these parameters are similar to those for swimming motor activity and if these parameters are relatively constant versus cycle time, as is the case for swimming (Wallén and Williams, 1984). Third, it is possible that pharmacological or electrical stimulation of isolated hemi-spinal cords activates locomotor networks in different ways than descending drive from locomotor command systems in the brain, a method that was used in the present study. Thus, bursting in isolated hemi-spinal cords in response to pharmacological or electrical stimulation might be an indication of the capabilities of spinal motor circuits under specific experimental conditions but not necessarily how they operate under normal conditions. This raises a significant

experimental dilemma, since it often is not known to what degree an experimental method that is convenient for eliciting rhythmic motor activity captures the critical features of the normal initiation of this activity. Fourth, there may be some differences regarding rhythmicity between the spinal locomotor CPGs in adult lamprey, which were examined in the two previous studies (Buchanan, 1999; Cangiano and Grillner, 2003), and those in larval lamprey, which were used in the present study. For example, the spinal CPGs in larval lamprey may be immature and lack certain features, or have different properties than those that are present in similar networks in adult animals (Cohen et al., 1990).

In *Xenopus* following midline lesions of the spinal cord, CPG modules in right and left sides of the cord are able to generate swimming-like motor activity, suggesting that these modules are rhythmogenic (Soffe, 1989). Furthermore, in paralyzed preparations, left-right alternating motor activity, typical of swimming, as well as occasional synchronous activity are observed (Kahn and Roberts, 1982), again suggesting that left and right CPG modules are rhythmogenic and left-right reciprocal connections largely control phasing of motor activity.

3. *Comparison to Studies in Limbed Animals*

Several studies with limbed invertebrates suggest that left and right CPG modules are rhythmogenic. In crayfish, right and left abdominal ganglia contain CPG modules that control the swimmerets and can function autonomously (Murchison et al., 1993). In *Clione*, right and left pedal ganglia not only can operate autonomously in controlling the right and left "wings", but within each ganglia neurons that generate dorsal and ventral

wing flexion activity can function in isolation as endogenous oscillators (reviewed in Arshavsky et al., 1998).

For limbed vertebrates, there are a number of studies suggesting that left and right spinal cord CPG modules are rhythmogenic. For example, left or right halves of the lower spinal cord, in the absence of left-right reciprocal connections, generate locomotor-like burst activity in a number of animals: mudpuppy (Cheng et al., 1998); embryonic chick (Ho and O'Donovan, 1993); neonatal mouse (Tao and Droge, 1992; Bonnot and Morin, 1998; Whelan et al., 2000; Bonnot et al., 2002); neonatal rat (Kudo and Yamada, 1987; Bracci et al., 1996; Cowley and Schmidt, 1997; Kjaefulff and Kiehn, 1997; Kremer and Lev-Tov, 1997; Nakayama et al., 2002); and cat (Kato, 1990). In addition, strychnine, which blocks left-right reciprocal inhibition, converts left-right alternating burst activity to synchronous bursting in the mudpuppy (Jovanovik et al., 1999) and neonatal rat (Cowley and Schmidt, 1995). Finally, during development in embryonic rat, motor patterns switch from left-right synchronous bursting to alternation as reciprocal connections between left and right halves of the spinal cord mature and change from excitation to inhibition (Nakayama et al., 2002).

In contrast to the above results, in turtle following removal of the left half of the lower spinal cord, stimulation of the right (left) receptive field for rostral scratching elicits rhythmic right hip flexor (extensor) bursts in the absence of antagonistic activity (Stein et al., 1995). Thus, in response to unilateral stimulation, contralateral spinal circuitry contributes to ipsilateral scratch motor pattern generation.

Experiments with limbed vertebrates suggest that flexor and extensor modules in spinal CPG networks are rhythmogenic. In neonatal rat lumbar spinal cord, application

of strychnine converts pharmacologically-elicited flexor-extensor alternation to synchronous bursting (Cowley and Schmidt, 1995), suggesting that flexor and extensor modules are rhythmogenic and reciprocal coupling largely controls the phasing of burst activity. However, as stated above for lamprey, synchronous burst activity in the presence of strychnine does not prove that the modules in question are rhythmogenic. In neonatal mouse spinal cord, pharmacologically elicited rhythmic flexor or extensor bursts can occur in the absence of antagonistic activity (Whelan et al., 2000). Furthermore, in the mudpuppy, surgically isolated flexor and extensor modules continue to generate pharmacologically-evoked rhythmic burst activity in the absence of reciprocal connections with their antagonistic modules (Cheng et al., 1998).

For the above studies in limbed vertebrates, pharmacological agents usually were applied to the isolated spinal cord to elicit spinal motor activity, and it is possible that these agents do not mimic all aspects of the normal initiation of rhythmic motor activity. In the turtle during sensory-evoked rostral scratch motor patterns, isolated hip flexion bursts sometimes can occur in the absence of ipsilateral hip extensor activity, (Currie and Gonsalves, 1999), and rhythmic synaptic potentials are absent in extensor motoneurons (Stein et al., 1982), suggesting a lack of activity in interneurons in the corresponding extensor module. These results suggest that hip-flexor modules are rhythmogenic and do not require reciprocal connections with hip-extensor modules. Similar approaches suggest that rhythmogenic knee-flexor and knee-extensor modules also are present in the spinal CPGs for scratching (Stein and Daniels-McQueen, 2004). Because the above variations of rostral scratch motor patterns were elicited in a relatively natural fashion by

sensory inputs, these results are perhaps the most convincing data that individual CPG modules can be rhythmogenic in some preparations.

In limbed vertebrates, the spinal CPGs that control a pair of limbs are thought to include right and left "half center" networks, each of which presumably includes a flexor and extensor module. However, the spinal locomotor networks controlling a single limb may be more complex and consist of multiple flexor-extensor half center networks, each of which controls flexor-extensor muscles acting around a different joint (hip, knee, ankle, etc.; Grillner, 1981). Thus, the rhythmicity of left and right half center networks or flexor and extensor modules in the spinal cords of limbed vertebrates may not be directly comparable to that of the spinal CPGs in the lamprey, which are thought to consist of a single half center network with left and right modules that are connected by reciprocal coupling.

4. *Conclusions*

In the present study, midline lesions were made in the spinal cords of larval lamprey to test the role of reciprocal coupling between left and right spinal CPG modules. Locomotor activity was initiated from the brain in both whole animals and *in vitro* preparations. The results suggest that isolated left and right hemi-spinal cords, in the absence of connections with intact spinal cord, do not function autonomously and do not generate rhythmic locomotor burst activity in response to descending activation from the brain. In *in vitro* preparations with a rostral midline spinal lesion, left and right hemi-spinal cords sometimes produced very slow burst activity, but the rhythmicity of this activity appeared to originate from the brain, and the parameters of the activity were

significantly different than those for normal swimming motor activity. In summary, in larval lamprey, reciprocal coupling, mediated by commissural interneurons, between left and right spinal CPG modules is not only important for left-right phasing of locomotor activity but also appear to contribute to rhythmogenesis.

Figure 33. Schematic diagram of proposed locomotor systems in the brain and spinal cord in lamprey. In the brain, locomotor command systems consist of five components: ventromedial diencephalon (VMD), dorsolateral mesencephalon (DLM), mesencephalic locomotor region (MLR), rostrolateral rhombencephalon (RLR), and reticulospinal (RS) neurons. RS neurons activate the spinal locomotor networks and initiate locomotor behavior. Trigeminal sensory inputs may initiate locomotor behavior by activating higher order centers (RLR) that then project to more rostral structures (DLM, VMD). The spinal locomotor networks are coupled together by a coordinating system to form a central pattern generator (CPG). Left and right oscillators are coupled by relatively strong reciprocal inhibition in parallel with weaker excitation.

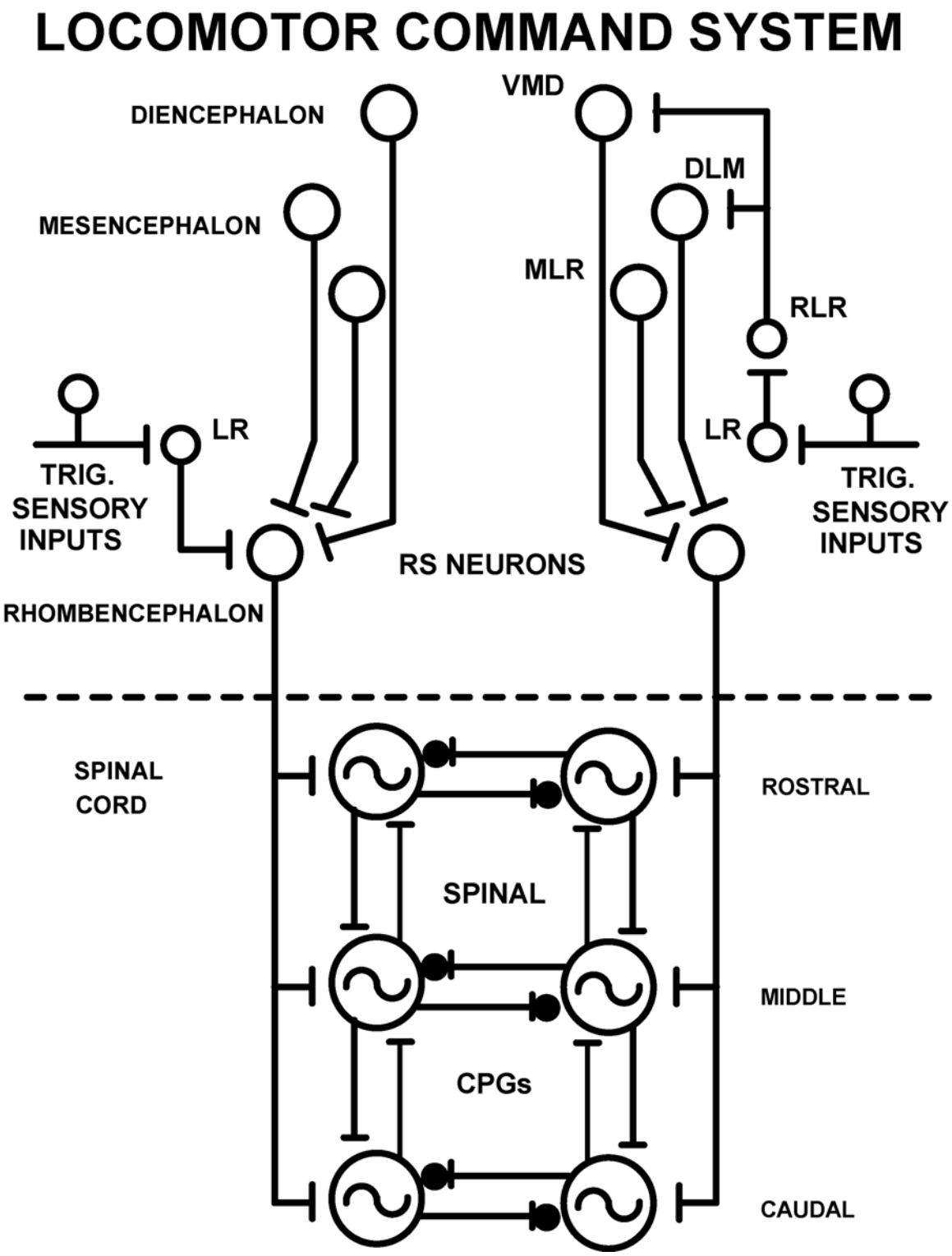
LOCOMOTOR COMMAND SYSTEM
DIENCEPHALON
VMD
DLM
MESENCEPHALON
MLR
RLR
LR
LR
TRIG.
SENSORY
INPUTS
TRIG.
SENSORY
INPUTS
RS NEURONS
RHOMBENCEPHALON
SPINAL
CORD
ROSTRAL
SPINAL
MIDDLE
CPGs
CAUDAL

Figure 33

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Vita

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