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LOCALIZATION, PHARMACOLOGY, AND ORGANIZATION OF BRAIN LOCOMOTOR AREAS IN LARVAL LAMPREY

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Abstract

In larval lamprey, spinal locomotor activity can be initiated by pharmacological microstimulation from the following higher order brain locomotor areas (Paggett et al., 2004; Jackson et al., 2007): rostralateral rhombencephalon (RLR); ventromedial diencephalon (VMD); or dorsolateral mesencephalon (DLM). In the present study, pharmacological microstimulation with excitatory amino acids (EAAs) or their agonists in the brains of *in vitro* brain/spinal cord preparations was used to determine the sizes, pharmacology, and organization of these locomotor areas. First, the RLR, DLM and VMD locomotor areas were confined to relatively small areas of the brain, and stimulation as little as 50 μ m outside these areas was ineffective or 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 ionotropic EAA receptor agonists could initiate spinal locomotor activity, but NMDA or AMPA was more reliable than kainate. 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 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 motor activity. Thus, the RLR locomotor area does not appear able to initiate spinal locomotor activity by neural circuits confined entirely within the rhombencephalon but requires more rostral neural centers, such as those in the VMD and DLM, as previously proposed (Paggett et al., 2004).

Keywords

locomotion; command system; reticulospinal; initiation; MLR

INTRODUCTION

In vertebrates, rhythmic locomotor behaviors are initiated by “command” systems in the brain that activate central pattern generators (CPGs) in the spinal cord to initiate rhythmic motor activity and locomotor movements (reviewed in Grillner, 1981). Reticulospinal (RS) neurons are the output neural elements of the command system (reviewed in Jordan, 1998). Electrical or pharmacological (chemical) microstimulation applied focally to several specific

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areas of the brain can initiate spinal locomotor activity (reviewed in Jordan, 1998). Presumably, at least some of the neurons in these “brain locomotor areas” are part of or have inputs to the locomotor command system. Electrical stimulation can activate a variety of neural elements near the stimulating electrode, including axons of passage. In contrast, pharmacological microstimulation, using neurotransmitters, agonists, or antagonists, is thought to activate cell bodies and dendrites but not, as a rule, axons of passage (Goodchild et al., 1982).

In a wide range of vertebrates, locomotor activity can be initiated by stimulation in similar brain locomotor areas, suggesting a common basic organization of locomotor command systems (reviewed in Grillner, 1981; McClellan, 1986; Jordan, 1998): (a) RS neurons in reticular nuclei (Steeves et al., 1987; Noga et al., 1988; Bernau et al., 1991); (b) mesencephalic locomotor region (MLR) (Shik et al., 1966; Kashin et al., 1974; Eidelberg et al., 1981; Parker and Sinnamon, 1983; Grillner and Wallen, 1984; Garcia-Rill and Skinner, 1987a,b; Bernau et al., 1991; Fetcho and Svoboda, 1993; Uematsu and Todo, 1997; Cabelguen et al., 2003); (c) subthalamic locomotor region (SLR) (Orlovsky, 1969,1970; Parker and Sinnamon, 1983; Skinner and Garcia-Rill, 1984); (d) pontomedullary locomotor strip (PLS) in the vicinity of the spinal nucleus of the trigeminal nerve (Mori et al., 1977; Kazennikov et al. 1981; Steeves et al., 1987; Beresovskii and Bayev, 1988; Bayev et al., 1988; Noga et al., 1988,1991); (e) lateral hypothalamus (Sinnamon, 1990); and (f) cerebellar locomotor region (CLR), which corresponds to the fastigial nucleus (Mori et al., 1999). The MLR and SLR project to reticular nuclei, and RS neurons are then thought to activate spinal CPGs (Orlovsky, 1970; Garcia-Rill and Skinner, 1987b).

In the lamprey, a lower vertebrate, spinal locomotor activity can be initiated by pharmacological or electrical microstimulation in several brain locomotor areas: reticular nuclei; the ventromedial diencephalon (VMD); the dorsolateral mesencephalon (DLM); and rostralateral rhombencephalon (RLR) (Hagevik et al., 1996; Hagevik, 1997; El Manira et al., 1997; Paggett et al., 2004; Jackson et al., 2007); and MLR (McClellan and Grillner, 1984; McClellan, 1989; Sirota et al., 2000). Many of the RS neurons that initiate locomotion project their descending axons primarily in the lateral parts of the spinal cord (McClellan, 1988; Shaw et al., 2010). In larval lamprey, DLM- or VMD-initiated locomotor activity usually is abolished or significantly attenuated when neural activity is blocked in reticular nuclei (Paggett et al., 2004), and RS neurons receive direct synaptic inputs from the VMD and DLM locomotor areas (El Manira et al., 1997; Paggett et al., 2004). In addition, RLR-initiated spinal locomotor activity often is abolished or markedly attenuated when neuronal activity is blocked in the DLM or VMD locomotor areas (Paggett et al., 2004). These results suggest that neurons in the RLR locomotor area project rostrally to the DLM and VMD areas, which then project to reticular nuclei (Paggett et al., 2004). Finally, pharmacological stimulation in dorsolateral parts of the rhombencephalon, in the vicinity of the descending trigeminal tract and sensory nucleus (Koyama et al. 1987), can sometimes initiate spinal locomotor activity (Hinton and McClellan, 1997; Hagevik, 1997).

Several aspects of the organization of the RLR, DLM, and VMD locomotor areas in lamprey remain to be investigated. First, although preliminary observations suggest that the DLM, RLR, and VMD locomotor areas are confined to relatively small regions of the brain (Hagevik et al., 1996), the actual sizes and shapes of these areas have not been determined in detail. Second, stimulation with EAAs in the above locomotor areas initiates spinal locomotor activity (Paggett et al., 2004; Jackson et al., 2007), but the contributions of the different types of ionotropic EAA receptors to the initiation of locomotion are not known. Third, it is not known if the RLR, DLM, or VMD locomotor areas can directly activate the spinal CPG networks. Fourth, during stimulation in the RLR, blocking neural activity in higher locomotor areas in the DLM or VMD often, but not always, abolishes or attenuates

initiated spinal locomotor activity (Paggett et al., 2004). Thus, the possibility remains that the RLR locomotor area might also initiate spinal locomotor activity via circuits confined to the rhombencephalon.

In the present study, mapping experiments were performed to determine the relative sizes and pharmacology 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 could be evoked by pathways entirely within the rhombencephalon or required more rostral neural centers, such as those in the VMD and DLM, as previously proposed (Paggett et al., 2004). Preliminary accounts of this work have appeared in abstract form (Hagevik et al., 1996; Jackson et al., 2006).

METHODS

Animal Care

Prior to *in vitro* experiments, larval sea lamprey (*Petromyzon marinus*) were maintained in ~10 L aquaria at 23–25°C. The procedures used in the present study were approved by the Animal Care and Use Committee at the University of Missouri.

Retrograde Labeling with HRP

Using *in vitro* brain/spinal cord preparations (Fig. 1A), stimulation sites in the brain were mapped and marked with a dye (see below, Fig. 1B). In order to provide anatomical references for these marked stimulation sites, prior to *in vitro* mapping experiments, descending brain neurons, ~80% of which are RS neurons (Davis and McClellan, 1994), were pre-labeled with horseradish peroxidase (HRP) (see Figs. 1B,2A,4). Approximately 10–14 days prior to setting up *in vitro* preparations (n = 23, 100–140 mm animal length), 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, normalized distance from head), and a Gelfoam pledget (Upjohn; Kalamazoo, MI) soaked in a 40% HRP (Type VI; Sigma Chemical) and 1% dimethyl sulphoxide solution was applied to the cut ends of the spinal cord, as previously described (Davis and McClellan, 1994; Zhang et al., 2002). Following tracer application, these animals were still able to swim quite effectively, even though muscle contractions would not be expected to occur in the caudal part of the body below the HRP application site (McClellan, 1990). Also, for *in vitro* brain/spinal cord preparations (see below), there were no obvious differences in spinal locomotor activity from animals with and without HRP labeling.

In the lamprey, trigeminal sensory inputs can elicit locomotor activity (McClellan, 1984), and these afferents synapse with second order neurons in the sensory nucleus of the descending trigeminal tract (Koyama et al. 1987) that eventually have inputs to brain locomotor command systems. Therefore, for experiments in which pharmacological stimulation was performed in the RLR locomotor area in the rhombencephalon (n = 13, 105–140 mm), ~3–5 days prior to setting up *in vitro* preparations, descending brain neurons (see above) as well as sensory axons in the descending trigeminal tracts were pre-labeled (dV, Fig. 1B; note that labeled trigeminal motoneurons were omitted for clarity). A ~3–4 mm transverse incision was made between the naris and anterior tip of the oral hood, and a small Gelfoam pledget soaked in HRP solution was placed in the incision site, as previously described (Calton et al., 1998). Following *in vitro* experiments, the brains were processed for HRP (see below).

In Vitro Brain/Spinal Cord Preparations

In vitro preparations from larval lamprey ($n = 37$, 93–140 mm) were set up as previously described in detail (Fig. 1A) (Hagevik and McClellan, 1994; McClellan, 1994; Paggett et al., 2004). Briefly, animals were anesthetized, most of the body musculature was removed, and the dorsal surface of the brain and spinal cord was exposed. The preparation was then pinned dorsal side up on the Sylgard-lined bottom (Dow Corning; Midland, MI) of a recording chamber containing oxygenated lamprey Ringer's solution (McClellan, 1990) maintained at 6–9°C. The choroid plexus was removed, the cerebellar commissure was transected, and the obex was extended slightly. Possible contractions of remaining musculature around the cranium and notochord were blocked by bath application of d-tubocurarine chloride (15 mg/l; Sigma Chemical). It should be noted that 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 in the present study) applied to the spinal cord (P. Hinton and A.D. McClellan, unpublished data). Thus, in the lamprey, bath-applied curare probably does not have a significant blocking effect on GABA_A receptors (Siebler et al., 1988), unlike the very strong disruption of spinal locomotor activity that occurs in the presence of bicuculline (Hagevik and McClellan, 1994).

For mapping and pharmacological experiments ($n = 23$), spinal locomotor activity in ventral roots was recorded with several suction electrodes (1,2 at segment 8–14, ~15–20% BL; 3 at segment 26–35, ~30–38% BL; Fig. 1A). In addition, the caudal end of the spinal cord was drawn into a suction electrode (SC in Fig. 1A at segment 40–56, ~42–55% BL;) to record spinal cord activity, particularly in those cases when pharmacological microstimulation was effective in activating neurons in the brain but did not initiate locomotor burst activity in spinal ventral roots (e.g. Fig. 2C4–2C6).

Stimulation in Brain Locomotor Areas

In the present study, bilaterally symmetrical pharmacological microstimulation (PE_R and PE_L, Fig. 1A) was used to identify the RLR, VMD, and DLM brain locomotor areas (Hagevik and McClellan, 1994; McClellan, 1994; Paggett et al., 2004), and to determine relative sizes, pharmacology, and organization of these brain locomotor areas. Two micropipettes were filled with 5 mM D-glutamate/5 mM D-aspartate (5 mM GLU/ASP) in lamprey Ringer's solution (pH = 7.2–7.4), and Fast green was added to visualize the ejection bolus (Hagevik and McClellan, 1994; McClellan, 1994). The above concentrations are about five times those typically used for bath application of these agents in lamprey experiments (Grillner et al. 1981; Rovainen, 1985) to compensate for the rapid decrease in agent concentration that occurs with distance from the tips of pressure ejection micropipettes (Stone, 1985). The tips of the micropipettes were broken off (~2–5 μ m tip dia.) and positioned bilaterally and symmetrically in one of the three brain locomotor areas ~25–50 μ m below the ventricular surface of the brain where many of the neuronal cell bodies are located (Nieuwenhuys and Nicholson, 1998). 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). In general, a single pressure pulse ejected a bolus with a radius of ~12.5–25 μ m (~8–65 pl), and 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 μ m (width of brain ~1 mm). Following each stimulation sequence and before stimulation was performed again in the same locomotor area, a period of at least 3 min. was allowed, as previously described (Hagevik and McClellan, 1994), during which the preparations were inactive. During an experiment, the recording chamber (~60 ml) was periodically changed with fresh Ringer's solution, and this procedure did not result in any obvious changes in spinal locomotor activity.

Mapping of Brain Locomotor Areas

To determine the sizes of the RLR, DLM, and VMD brain locomotor areas, the tips of the stimulation pipettes were systematically moved in and around these areas. Because very different responses could be elicited from stimulation sites that were separated by $\leq 50 \mu\text{m}$, substantial diffusion of stimulating agents and activation of neurons that were distant from the stimulation site was unlikely. Following stimulation at a particular site in the brain, a small amount of Fast green remained temporarily at the stimulation site. A separate manipulator was then used to position the tip of a different micropipette containing 1–3% Alcian blue (Sigma Chemical) in Ringer's solution at the stimulation 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 the technique of marking stimulation sites with electrolytic lesions, which might affect the responses elicited by subsequent pharmacological microstimulation in nearby brain areas. During experiments, all stimulation sites were marked on a diagram of the brain so that these sites could be matched to dye-marked sites after the experiment. After testing and marking one stimulation site in the brain, the tips of the stimulating micropipettes were moved $\sim 50 \mu\text{m}$ 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 spinal locomotor activity (see below) and a circumscribed region around the locomotor area that was ineffective or elicited uncoordinated ventral root activity (see Fig. 2B). After histological processing of the brains (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 (see below).

Pharmacology of Locomotor Areas

Brain locomotor areas were located using 5 mM GLU/ASP, after which the pharmacology was determined for the most effective site within each area (i.e. “best sites”) that appeared to initiate locomotor activity with the shortest and least variable cycle times. Specifically, the types of excitatory amino acid (EAA) receptors present at the “best site” for each locomotor area were determined by pressure ejection of each of the following agents (Carpenter and Dickerson, 2001): (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 GLU/ASP, the concentrations for NMDA and KA were approximately five times the typical bath concentrations used for lamprey experiments (Grillner et al. 1981; Rovainen, 1985), while the concentration for AMPA was taken from previous pressure ejection studies (El Manira et al., 1997). Alcian blue was used to bilaterally mark each of the stimulation sites.

Organization of Locomotor Command System

Results from our previous study suggest that neurons in the RLR project to locomotor areas in the DLM and VMD, and these latter areas activate RS neurons (Paggett et al., 2004), which are involved in initiation of spinal locomotor activity (Jackson et al., 2007). In the present study, two types of experiments were performed to examine further the organization of certain parts of the locomotor command system. First, for some *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. 7A). Spinal locomotor activity in ventral roots was recorded with several suction (1,2 at segment 16–27, ~ 22 –31% BL; 3 at segment 40–49, ~ 42 –49% BL; Fig. 7A). In addition, the caudal end of the spinal cord was drawn into a suction electrode (SC in Fig. 7A at segment 58–83, ~ 57 –78% BL;) to

record spinal cord activity. Spinal locomotor activity was elicited by pressure ejection of 5 mM GLU/ASP in the VMD, DLM, or RLR locomotor areas. Subsequently, synaptic transmission was blocked in the brain by application of a zero calcium Ringer's solution containing 2 mM MnCl_2 (McClellan, 1984) to the brain pool (Pool I), and pharmacological stimulation was repeated for the same locomotor areas. 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. It should be noted that with synaptic transmission blocked in the brain of semi-intact preparations, pharmacological stimulation with GLU/ASP in reticular nuclei still is effective in activating RS neurons and initiating swimming movements of the body (Jackson et al., 2007).

Second, in a different set of experiments ($n = 3$), pharmacological microstimulation in the RLR with 5 mM GLU/ASP was used to initiate spinal locomotor activity. Subsequently, the stimulation pipettes were withdrawn, and a complete transection was made at the mesencephalic-rhombencephalic border with iridectomy scissors (Fig. 8A). Care was taken to prevent stretching of the trigeminal roots during the transection. Stimulation in the RLR was repeated to test whether spinal locomotor activity could be initiated via brain neural circuits confined entirely to 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; LeRay et al., 2004).

Data Recording and Analysis

Ventral root activity was rectified and integrated ($\tau = 50$ ms) to better reveal locomotor bursts. Brain-initiated locomotor and spinal cord activity (Fig. 2) were stored on tape (Neuro-Data DR890; Cygnus Technologies, Delaware Water Gap, PA; 11 kHz sampling rate per channel). Five criteria were used to define chemical microstimulation that initiated "good quality" locomotor activity (see Fig. 2C1–2C3, Tables 1–3): (a) stimulation with a given agent had to initiate at least ten cycles of locomotor activity, but almost all locomotor episodes included many 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 1,3); (c) stimulation at a given site in the brain had to initiate locomotor activity during at least two consecutive trials; (d) *in vitro* spinal locomotor activity had to consist of left-right alternation of burst activity at the same segmental level ($1 \leftrightarrow 2$) as well as a rostrocaudal phase lag of ipsilateral locomotor burst activity ($2 \rightarrow 3$) (see below and Fig. 1C); and (e) the cycle times (T , see below) of locomotor activity had to be less than 3 s (McClellan, 1994), but most cycle times were substantially shorter.

Locomotor activity was acquired with a custom data acquisition and analysis system, with which the onsets and offsets of locomotor burst activity were marked and imported into a spreadsheet program to calculate various locomotor parameters (McClellan and Hagevik, 1997): cycle times (T) were defined as the interval between 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 ($2 \rightarrow 3$, Fig. 1C) and cycle time, divided by the number of intervening segments; and right-left phase values ($\phi_{\text{RT-LT}}$) were calculated as the phase of the midpoints of right locomotor burst activity within cycles defined by the midpoint of left locomotor burst activity (Fig. 1C).

Histological Processing

For those *in vitro* preparations in which HRP was applied prior to the experiments ($n = 23$), following the experiments the brains and spinal cords were processed for HRP using a

modification of the Hanker-Yates protocol, as previously described (Davis and McClellan, 1994; Zhang et al., 2002). Subsequently, the tissue was dehydrated in an ethanol series and cleared in methyl salicylate, and whole mounts were prepared on slides with Permount (Fisher Scientific; Fair Lawn, NJ). 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. 1B). 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 different focal plane. Also, the positions of Alcian blue-labeled stimulation spots were entered into the marking/tracing system (see Fig. 2A,B). The marking/tracing system produced two-dimensional diagrams of the brains as if all structures, HRP-labeled neural elements, and marked stimulation sites were in a single plane of focus (see Figs. 1B, 2A,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 (e.g. see Figs. 2A,B and 3A for VMD locomotor area). Because the whole mount tissue was relatively flat, dorsal (ventral) areas of the brain appear lateral (medial) in the brain diagrams (e.g. Fig. 1B). Finally, in the whole mount tissue, the widths (transverse axis) and lengths (rostrocaudal axis) of brain locomotor areas were measured (Table 2). Previous studies indicate that the degree of tissue shrinkage in the brain from the histological processing used in the current study is less than ~5% (Hagevik and McClellan, unpublished observation).

Statistics

For each preparation, the spot within a brain locomotor area (RLR, DLM, VMD) at which 5 mM GLU/ASP initiated locomotor activity with the shortest and least variable cycle times was defined as the “best site” (see Methods). The parameters of spinal locomotor activity for the “best site” within each brain locomotor area and for each pharmacological agent (e.g. Fig. 2C1) were averaged (mean $\pm$ SD) and compared using one-way ANOVA with Bonferroni multiple comparisons post-test (Tables 1,3). Values were considered to be statistically significant for $P \leq 0.05$.

RESULTS

Extent and Pharmacology of Brain Locomotor Areas

Pharmacological microstimulation in the VMD—In successive stimulation trials, bilateral pharmacological microstimulation with 5 mM D-glutamate/5 mM D-aspartate (5 mM GLU/ASP) in a restricted brain region in the ventromedial diencephalon (VMD, Fig. 2A,B) reliably initiated well-coordinated spinal locomotor activity ($n = 5$, Fig. 2C1–2C3). Brain-initiated ventral root activity consisted 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 ($2 \rightarrow 3$, Fig. 2C1–2C3; Table 1), as previously described (Hagevik and McClellan, 1994). For the most part, the parameters of *in vitro* locomotor activity initiated from the VMD were not significantly different than those for muscle burst activity during swimming in whole animals (Table 1). Cycle times for VMD-initiated *in vitro* spinal locomotor activity were significantly longer than those for muscle locomotor burst activity in whole animals (T, Table 1). However, these general 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 than *in vitro* preparations.

The relative size of the VMD locomotor area varied to some extent in different preparations (Fig. 2B, Table 2), but the size did not appear to be correlated with the cycle times of initiated locomotor activity (see Discussion). However, in all preparations ($n = 5$) the borders of the VMD locomotor area were 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. 2B; see below). In the preparation illustrated, movement of the stimulation pipettes ~50 μ M lateral (C2) or rostral (C3) to the most effective stimulation spot (Fig. 2C1) still initiated well-coordinated spinal locomotor activity, albeit with a slight reduction in the amplitudes of some bursts (Fig. 2C2, 2C3). In contrast, stimulation as little as 50 μ M outside of the VMD locomotor area did not initiate spinal locomotor activity (Fig. 2B, 2C4–2C6), strongly suggesting that pressure ejection focally activated neurons relatively close to the tips of the stimulation pipettes. Interestingly, even for stimulation sites that were ineffective, there still was substantial activity recorded from the caudal end of the spinal cord (SC in Fig. 2C4–2C6). This suggests that descending brain neurons were still activated, but not appropriately or sufficiently to activate spinal CPGs.

In all preparations, well-coordinated spinal locomotor activity could be reliably initiated from the VMD locomotor area ($n = 3$, Fig. 3A) by pharmacological microstimulation with all the agents tested: 5 mM GLU/ASP (Fig. 3B1); 0.5 mM NMDA (Fig. 3B2); 1 mM AMPA (Fig. 3B3); or 0.25 mM KA (Fig. 3B4). Thus, in larval lamprey, both NMDA and non-NMDA glutamate receptors are present in this locomotor area and probably participate in VMD-initiated spinal locomotor activity. Finally, there were no significant differences in the parameters of spinal locomotor activity initiated from the VMD by the different agents (Table 3).

Pharmacological microstimulation in the DLM—Pharmacological microstimulation with 5 mM GLU/ASP within a restricted region in the dorsolateral mesencephalon (DLM, Fig. 4A) reliably initiated well-coordinated spinal locomotor activity ($n = 5$). Similar to that discussed above for the VMD locomotor area, except for cycle times, the parameters for DLM-initiated locomotor activity were not significantly different than those for muscle burst activity during swimming in whole animals (Table 1). Also, the parameters of DLM- and VMD-initiated locomotor activity, including cycle times, were not statistically different (Table 1).

The boundaries of the DLM locomotor area were formed caudally by the mesencephalic-rhombencephalic border, and dorsolaterally by the lateral tectal recess (Neuwenhuys and Nicholson, 1998), and ventrally by stimulation sites outside the DLM (Fig. 4A). The DLM locomotor area often was somewhat crescent-shaped, rather than rectangular, and tended to course adjacent to the lateral tectal recess in the basal plate. As was the case for the VMD, the relative size of the DLM locomotor area varied to some extent in different preparations (Table 2) but did not appear to be correlated with cycle times. In all experiments, the rostral and ventral borders of the DLM were formed by a clear separation between stimulation sites that initiated spinal locomotor activity and relatively nearby stimulation sites surrounding the DLM that were ineffective or elicited uncoordinated ventral root burst activity (Fig. 4A).

Pharmacological microstimulation in the DLM locomotor area ($n = 3$; squares in Fig. 5A) with 5 mM GLU/ASP (Fig. 5B1), 0.5 mM NMDA (Fig. 5B2), 1 mM AMPA (Fig. 5B3), or 0.25 mM KA (Fig. 5B4) initiated 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 in the VMD with the different agents (Table 3).

Pharmacological microstimulation in the RLR—In successive trials, stimulation with 5 mM GLU/ASP in the RLR locomotor area (Fig. 4B) reliably initiated well-coordinated spinal locomotor activity ($n = 5$). Except for cycle times, the parameters of RLR-initiated ventral root burst activity were not significantly different than those during swimming in

whole animals (Table 1). Furthermore, the parameters of spinal locomotor activity initiated by stimulation in the RLR were not significantly different than those elicited by stimulation in the VMD or DLM (Table 1).

The relative size of the RLR locomotor area varied to some extent in different preparations (Fig. 4B, Table 2), as described for the VMD and DLM locomotor areas. The borders of the RLR locomotor area were 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. 4B). The RLR locomotor area was somewhat crescent-shaped, rather than rectangular, and tended to course adjacent to the sulcus limitans in the basal plate.

In all preparations, pharmacological microstimulation in the RLR (triangles, Fig. 6A) with 5 mM GLU/ASP reliably initiated well-coordinated spinal locomotor activity (Fig. 6B1). Microstimulation with 0.5 mM NMDA (Fig. 6B2) or 1 mM AMPA (Fig. 6B3) initiated spinal locomotor in most preparations (4/6 for NMDA and 6/7 for AMPA). However, for successive stimulation trials with NMDA or AMPA, there usually was a progressive increase in the onset latency of locomotor activity, and the burst activity became slightly more erratic (data not shown). In contrast, stimulation with 0.25 mM kainate in the RLR locomotor area (Fig. 6B4) initiated spinal locomotor activity in only some preparations (2/6 animals), while in the remaining preparations (4/6 animals), stimulation resulted in uncoordinated ventral root burst activity that was reduced in amplitude with repeated stimulation trials (data not shown). Finally, there were no significant differences in the parameters of spinal locomotor activity initiated from the RLR by the different agents (Table 3) or compared to those initiated from the VMD or DLM (Table 3).

Organization of Higher Order Locomotor Areas

First, in partitioned *in vitro* brain/spinal cord preparations ($n = 11$; Fig. 7A, see Methods), spinal locomotor activity was reliably initiated by pharmacological microstimulation with 5 mM GLU/ASP in the VMD (Fig. 7B1), DLM (Fig. 7C1), or RLR (Fig. 7D1). Subsequently, after bath application of a zero calcium Ringer's solution (see Methods) to the brain pool (Pool I, Fig. 7A) to locally block synaptic transmission, stimulation in the VMD (Fig. 7B2), DLM (Fig. 7C2), or RLR (Fig. 7D2) no longer initiated ventral root activity (electrodes 1–3). Furthermore, no activity was recorded from the caudal end of the spinal cord (SC in Fig. 7B2–7D2). Under these conditions, there was no indication of descending activation of the spinal cord from the brain, and this strongly suggests that the three brain locomotor areas do not directly activate spinal locomotor networks (see Discussion).

Second, in control *in vitro* preparations prior to brain lesions (Fig. 8A), pharmacological microstimulation in the RLR locomotor area reliably initiated spinal locomotor activity ($n = 3$; Fig. 8B1). In addition, brief mechanical stimulation applied to the oral hood elicited some uncoordinated burst activity in ventral roots and tonic activity recorded from the caudal end of the spinal cord (Fig. 8B2). Following a complete transection at the mesencephalic-rhombencephalic border (thick lines, Fig. 8A), pharmacological microstimulation in the RLR locomotor area elicited virtually no ventral root activity but did evoke some tonic activity from the caudal end of the spinal cord (Fig. 8C1). These results suggest that spinal locomotor activity could not be initiated from the RLR via neural circuits confined entirely to the rhombencephalon. Under these conditions, the tonic activity in the caudal spinal cord that was initiated by RLR stimulation might be due to activation of a few RS neurons in the rostral ARRN (Fig. 8A). Following the above brain lesion, brief mechanical stimulation applied to the oral hood still elicited some uncoordinated ventral root bursts as well as

caudal spinal cord activity (Fig. 8C2), suggesting that stimulation of trigeminal afferents could still result in activation of RS neurons (LeRay et al., 2004).

DISCUSSION

Before summarizing the results from the present study, several technical issues should be mentioned. 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 pharmacological microstimulation appeared to activate neural structures in the brain that were relatively close to the tips of stimulating micropipettes, and movement of the stimulating micropipettes by as little as 50 μm could result in dramatically different evoked spinal motor activity or no elicited activity (e.g. Fig. 2C2 and 2C4). In addition, preliminary results indicate that during intracellular recording from large, identified RS neurons, continuous pressure ejection of 5 mM GLU/ASP could be as close as 75–100 μm to the soma before depolarizing potentials and action potentials were elicited (unpublished observations). Together, these results suggest that in the present study, the neural structures activated by pharmacological microstimulation were confined to discrete regions of the brain (see Table 2) that were relatively close to the tips of the stimulating pipettes.

Size, Pharmacology, and Organization of Higher Order Locomotor Areas

In a previous study using semi-intact preparations from larval lamprey, it was shown that pharmacological microstimulation in the VMD, DLM, or RLR brain locomotor areas, as well as in reticular nuclei, with 5 mM GLU/ASP initiates locomotor movements and locomotor muscle activity that are very similar to those during swimming in whole animals (Jackson et al., 2007). In the present study, *in vitro* brain/spinal cord preparations and bilateral pharmacological microstimulation in higher locomotor areas (VMD, DLM, or RLR; Hagevik and McClellan, 1994; McClellan, 1994; Paggett et al., 2004) were used to investigate four aspects of higher order locomotor areas in the lamprey brain: relative sizes of locomotor areas; pharmacology of neurons in these areas; test of whether these locomotor areas could directly activate the spinal CPGs; and test of whether the RLR could initiate spinal locomotor activity via circuits confined to the rhombencephalon. First, stimulation in the DLM, VMD, or RLR locomotor areas initiated well-coordinated spinal locomotor activity, and these locomotor areas were restricted to relatively small areas of the brain, suggesting that initiation of spinal locomotor activity was not due to widespread stimulation of nonspecific neural elements. However, the sizes of effective brain locomotor areas varied slightly in different preparations and were not correlated to cycle times of initiated locomotor activity (Table 2). Presumably these slight variations are due to some differences in distributions of neuronal cell bodies and/or dendrites within locomotor areas, and possibly some variation in the volumes and precise locations of ejected agents. In contrast, in decerebrate cats, the size of the MLR is larger for evoked treadmill locomotion (i.e. intact spinal sensory inputs) compared to that for fictive locomotion (Amemiya and Yamaguchi, 1984). Thus, in some cases the effective sizes of brain locomotor areas appear to vary with the general excitability of the nervous system.

Second, pharmacological microstimulation in the DLM and VMD with NMDA, AMPA, or kainate reliably initiated spinal locomotor activity, suggesting that NMDA and non-NMDA EAA receptors are present in these locomotor areas. In the RLR, stimulation with NMDA and AMPA initiated locomotor activity in most preparations, while stimulation with kainate was less reliable. These differences in effectiveness of agents applied to the RLR presumably are due to differences in the relative densities of the various ionotropic EAA receptors.

Third, when synaptic transmission was blocked in the brain, stimulation in the RLR, DLM, or VMD did not elicit any detectable spinal ventral root activity or activity at the caudal end of the spinal cord (Fig. 7). In addition, these brain locomotor areas are not in close proximity to substantial numbers of descending brain neurons (Fig. 1B). Together, these results strongly suggest that neurons in these locomotor areas do not directly activate spinal locomotor networks.

Fourth, following a transection at the mesencephalic-rhombencephalic border, stimulation in the RLR locomotor area no longer initiated *in vitro* spinal motor activity (1–3 and SC; Fig. 8) via neural circuits confined entirely within the rhombencephalon (Fig. 8). Also, in whole animals, a transection at the mesencephalic-rhombencephalic border blocks trigeminal-evoked locomotor behavior (McClellan, 1988). These results appear to raise some concerns regarding the suggestion that in the lamprey, trigeminal sensory inputs elicit escape swimming via a disynaptic pathway entirely within the rhombencephalon (Viana Di Prisco et al., 1997; LeRay et al., 2004).

Possible Neural Substrates for Brain Locomotor Areas

Although the anatomical identification of the VMD, DLM, and RLR locomotor awaits histological analysis, some insights can be obtained from previous studies. In larval lamprey, the VMD locomotor area (Paggett et al., 2004; Jackson et al., 2007; present study) probably corresponds to the “diencephalic locomotor region” (DLR) in the ventral thalamus of adult animals (El Manira et al., 1997). In semi-intact preparations from larval and adult lamprey, stimulation in this brain area initiates swimming movements (Jackson et al., 2007; Ménard and Grillner, 2008). This area of the brain receives inputs from several sources, including olfactory bulbs as well as the tectum and isthmus region (El Manira et al., 1997).

The DLM locomotor area is located in the dorsolateral basal plate of the mesencephalon (Fig. 1 in Paggett et al., 2004), adjacent to the lateral tectal recess, which is the approximate border between the optic tectum and torus semicircularis (González et al. 1999; Neuwenhuys and Nicholson, 1998). In semi-intact preparations of larval and adult lamprey, stimulation in this area of the brain initiates swimming movements (Jackson et al., 2007; Saitoh et al., 2007). The tectum receives a number of types of inputs (de Arriba and Pombal, 2007), including the sensory nucleus of the descending trigeminal tract that contains second order trigeminal sensory neurons, some of which initiate escape swimming (McClellan, 1984), and neurons in the dorsal column nuclei, some of which appear to initiate locomotion (Grillner and McClellan, 1983). However, some of the stimulation sites in the DLM appear to be in the torus semicircularis (Fig. 4A2), which also receives input from the sensory nucleus of the descending trigeminal tract and projects to reticular nuclei (González et al. 1999), presumably for initiating motor responses.

In the larval lamprey brain, the RLR locomotor area is located in the dorsolateral basal plate of the rhombencephalon (Fig. 1 in Paggett et al., 2004), adjacent to the sulcus limitans. This brain area is in the general region of the rostral extent of the trigeminal descending tract (dV; Fig. 4B) and sensory nucleus as well as the isthmus nucleus (González et al., 1999). Although the RLR locomotor area is relatively close to the anterior ARRN (Fig. 4B), which also can initiate locomotor activity (Jackson et al., 2007), these very likely are separate locomotor areas. For example, in semi-intact preparations in which synaptic transmission is blocked in the brain, pharmacological stimulation in parts of the ARRN initiates swimming movements (Jackson et al., 2007), indicating that neurons in the ARRN can directly activate spinal locomotor networks. In contrast, under similar conditions stimulation in the RLR no longer initiated detectable spinal ventral root activity or activity at the caudal end of the spinal cord (Fig. 7). Also, RLR-initiated locomotor activity often is abolished or attenuated by blocking neural activity in the DLM or VMD, suggesting that unlike RS neurons in the

ARRN, at least some of the neurons in the RLR locomotor area project rostrally (Paggett et al., 2004). Finally, results from the present study indicate that there is an area between the RLR and effective sites in the ARRN that does not initiate spinal locomotor activity (Fig. 4B).

Relationship between Brain Locomotor Areas in the Lamprey

Pharmacological stimulation in reticular nuclei (ARRN, MRRN, and PRRN) results in direct activation of spinal locomotor networks and drives locomotor burst activity (Jackson et al., 2007). Many of the RS neurons in these nuclei project in the lateral spinal tracts, which are important for the initiation of locomotion (McClellan, 1988; Shaw et al., 2010).

Brief electrical stimulation in the VMD (DLR), DLM, and MLR locomotor areas elicits monosynaptic responses in RS neurons that can summate (Paggett et al., 2004; also see El Manira et al., 1997; McClellan, 1989; Sirota et al., 2000). Also, blocking experiments support the notion that the RLR locomotor area projects to the VMD and DLM areas, which then project to reticular nuclei (Paggett et al., 2004).

Although RS neurons appear to be the main output neural elements of the brain locomotor command system, it is unclear why there are at least four higher order brain locomotor areas that can reliably initiate spinal locomotor activity. Perhaps some of these areas are synaptically connect as part of an initiation pathway, such as suggested above for the RLR, DLM, and VMD. In addition, some of the locomotor areas may preferentially participate under certain conditions or in response to particular sensory inputs. In mammals, similar explanations have been proposed regarding the different higher order locomotor areas (MLR, SLR, PLS, CLR, and lateral hypothalamus), but it is not entirely clear how they interact during and contribute to the natural initiation of locomotion (see Fig. 1 in Jordan, 1998).

Other Brain Locomotor Areas in the Lamprey

Neurons in the lamprey MLR also project to reticular nuclei and elicit monosynaptic responses in RS neurons (McClellan, 1989; Sirota et al., 2000). In *in vitro* brain/spinal cord preparations, electrical stimulation in this brain 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 increases the frequency of swimming activity (McClellan and Grillner, 1984; Sirota et al., 2000).

Comparison to Brain Locomotor Areas in Other Vertebrates

The general organization of brain locomotor areas in the lamprey appears to share some similarities with those in “higher” vertebrates. First, in cat, activation of trigeminal afferents (Aoki and Mori, 1981) or stimulation of the PLS, initiate locomotor activity (Mori et al., 1977; Kazennikov et al. 1981; Steeves et al., 1987; Beresovskii and Bayev, 1988; Noga et al., 1988,1991). The PLS corresponds to the spinal nucleus of the trigeminal nerve (Bayev et al., 1988; Beresovskii and Bayev, 1988; Noga et al., 1988,1991), which contains second order trigeminal sensory neurons and is analogous to the nucleus of the descending trigeminal tract in lamprey. Second, in mammals, the subthalamic locomotor region (SLR) does not project directly to spinal locomotor networks but activates RS neurons, which then initiate locomotion (Orlovsky, 1969; Parker and Sinnamon, 1983; Skinner and Garcia-Rill, 1984). Third, the MLR also does not directly activate spinal locomotor networks but instead initiates locomotion via activation of RS neurons (Orlovsky, 1970; Garcia-Rill and Skinner, 1987a,b; reviewed in Jordan, 1998). Finally, RS neurons thought to be important for initiating locomotion are located in the medioventral medulla, project in ventrolateral spinal

tracts, and directly activate spinal locomotor networks (Steeves et al., 1987; Noga et al., 1988; reviewed in Jordan, 1998). In particular, lesions or injection of pharmacological blockers in these reticular nuclei abolish MLR-elicited locomotor activity (Garcia-Rill and Skinner, 1987a; Bernau et al., 1991; Noga et al., 1991).

SUMMARY

For *in vitro* brain/spinal cord preparations from larval lamprey, pharmacological microstimulation in the DLM, VMD, or RLR initiated well-coordinated spinal locomotor activity. Similar to brain locomotor areas in mammals, those in the lamprey are restricted to relatively small, discrete areas of the brain. In the lamprey, neurons in the above brain locomotor areas appear to have NMDA as well as non-NMDA EAA receptors. Finally, these locomotor areas do not directly activate spinal locomotor networks, and RLR-initiated locomotor activity requires more rostral neural circuits and not just pathways confined entirely to the rhombencephalon.

Research Highlights

- the RLR, DLM and VMD locomotor areas were confined to small brain areas
- NMDA and non-NMDA receptors are present in the brain locomotor areas
- the locomotor areas do not directly activate spinal locomotor networks.
- the RLR locomotor area requires more rostral neural pathways in the brain

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FIGURE 1.

(A) *In vitro* brain/spinal cord preparation showing bilateral pharmacological microstimulation pipettes (PE_R and PE_L), ventral root recording electrodes (1, 2, and 3), and electrode at the caudal end of the spinal cord (SC) (see Methods for segmental locations). (B) Diagram of dorsal view of brain (left) and rostral spinal cord (right) with traces around cell groups containing descending brain neurons, symbols representing brain locomotor areas, and infundibulum (shaded ellipse). Locomotor areas: DLM (squares) - dorsolateral mesencephalon; MLR (open circles) - mesencephalic locomotor region; RLR (triangles) - rostromedial rhombencephalon; and VMD (filled circles) - ventromedial diencephalon. Anatomical abbreviations: ALV - anterolateral vagal group; ARRN - anterior

rhombencephalic reticular nucleus; Di - diencephalon; DLV - dorsolateral vagal group; MRN - mesencephalic reticular nucleus; MRRN - middle rhombencephalic reticular nucleus; PLV - posterolateral vagal group; PON - posterior octavomotor nucleus; PRRN - posterior rhombencephalic reticular nucleus; (modified from Davis and McClellan 1994, Paggett et al., 2004, and Sirota et al., 2000). Also shown are traces around the descending trigeminal tracts (dV; note that the trigeminal motor nuclei have been omitted for simplicity). (C) Bilateral microstimulation (PE = pressure ejection pulses) with 5 mM D-glutamate/5 mM D-aspartate (GLU/ASP) in the (C1) VMD, (C2) DLM, and (C3) RLR initiated spinal locomotor activity, 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, neural activity also was recorded from the caudal end of the spinal cord (SC).

FIGURE 2.

(A) Diagram of brain showing traces around reticular nuclei (MRN, ARRN, MRRN, PRRN) and infundibulum (shaded ellipse) as well as stimulation sites in the VMD locomotor area (filled circles) that initiated spinal locomotor activity as well as ineffective sites outside the VMD 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 matched recordings in C. (C) Spinal locomotor activity (1–3) and caudal spinal cord activity (SC) (see legend in Fig. 1A,C) initiated by pharmacological microstimulation (PE = pressure ejection pulses) with 5 mM GLU/ASP within the VMD (C1–C3; filled circles in A,B), and uncoordinated burst activity initiated by stimulation in sites outside the VMD (C4–C6; open circles in A,B).

FIGURE 3.

Pharmacology of VMD locomotor area. (A) Diagram of brain showing reticular nuclei and stimulation sites in the VMD (filled circles). (B) Spinal locomotor activity (1–3) and caudal spinal cord activity (SC) (see legend in Fig. 2) initiated by pharmacological microstimulation (PE) in the VMD locomotor area with (B1) 5 mM GLU/ASP, (B2) 0.5 mM NMDA, (B3) 1.0 mM AMPA, or (B4) 0.25 mM KA.

FIGURE 4.

(A1) Brain diagram showing stimulation sites in and around the DLM locomotor area (see legend in Fig. 2). (A2) Enlarged view of left side of brain showing stimulation sites within the DLM that initiated spinal locomotor activity (filled circles) and sites just outside the DLM that were ineffective (open circles). (B1) Diagram of brain showing reticular nuclei and descending trigeminal tracts (dV; see Fig. 1B) as well as stimulation sites in and around the RLR locomotor area (see legend in Fig. 2). (B2) Enlargement of left side of brain showing stimulation sites within the RLR that initiated spinal locomotor activity (filled circles) as well as sites outside the RLR that elicited uncoordinated activity (open circles).

FIGURE 5.

Pharmacology of DLM locomotor area. (A) Diagram of brain showing reticular nuclei and bilateral stimulation sites in the DLM (squares). (B) Locomotor activity initiated by stimulation in the DLM locomotor area with (B1) GLU/ASP, (B2) NMDA, (B3) AMPA, or (B4) KA (see legend in Fig. 3).

FIGURE 6. Pharmacology of RLR locomotor area. (A) Brain diagram showing reticular nuclei and descending trigeminal tracts (dV) as well as bilateral stimulation sites in the RLR (triangles). (B) Locomotor activity initiated by stimulation in the RLR locomotor area with (B1) GLU/ASP, (B2) NMDA, (B3) AMPA, or (B4) KA (see legend in Fig. 3).

FIGURE 7.

(A) Diagram of partitioned *in vitro* brain/spinal cord preparation with pharmacological microstimulation pipettes (PE_R and PE_L), ventral root recording electrodes (1, 2, and 3), electrode at caudal end of the spinal cord (SC), and Vaseline-sealed barrier (thick 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 stimulation with 5 mM GLU/ASP in the VMD, DLM, or RLR locomotor areas. (B2–D2) After applying a zero calcium Ringer's solution to Pool I to block chemical synaptic transmission in the brain, stimulation in the above brain locomotor areas no longer elicited ventral root burst activity (1–3) or activity from the caudal end of the spinal cord (SC).

FIGURE 8.

(A) Brain diagram with traces around reticular nuclei (see Fig. 1B), bilateral stimulation sites in the RLR locomotor area (triangles), and location of transection at the mesencephalic-rhombencephalic border (thick diagonal lines). (B) Prior to making a lesion at the mesencephalic-rhombencephalic border. (B1) Stimulation in the RLR initiated spinal locomotor activity consisting of left-right alternation of burst activity ($1 \leftrightarrow 2$) and a rostrocaudal phase lag ($2 \rightarrow 3$). (B2) Brief mechanical stimulation of the left side of the oral hood (triangle) elicited some ventral root (1–3) and caudal spinal cord (SC) activity. (C) Following a transection at the mesencephalic-rhombencephalic border (see A; same animal as B). (C1) Stimulation in the RLR locomotor area no longer initiated spinal locomotor burst activity (1–3) but still evoked activity recorded from the caudal end of the spinal cord (SC). (C2) Mechanical stimulation of the left side of the oral hood (triangle) still elicited some weak ventral root activity (1–3) and activity recorded from the caudal spinal cord (SC).

TABLE 1

PARAMETERS OF LOCOMOTOR ACTIVITY INITIATED BY PHARMACOLOGICAL MICROSTIMULATION^a IN DIFFERENT BRAIN LOCOMOTOR AREAS^b

	T (ms)	BProstral	BPcaudal	φ _{RT-LT}	φ _{INT}	LATENCY (sec)
Whole Animals ^c	342 ± 79 7/595 ^d	0.373 ± 0.075 7/595	0.361 ± 0.081 7/595	0.520 ± 0.100 10/367	0.006 ± 0.002 7/595	--
Locomotor Area						
VMD	1095 ± 313 * 5/417	0.334 ± 0.063 5/417	0.326 ± 0.108 5/417	0.523 ± 0.099 5/417	0.003 ± 0.003 5/417	11 ± 4 (5–17) ^e
DLM	1470 ± 531 ** 5/322	0.269 ± 0.080 5/322	0.283 ± 0.116 5/322	0.490 ± 0.067 5/322	0.004 ± 0.003 5/322	12 ± 7 (6–29)
RLR	1117 ± 351 * 5/438	0.307 ± 0.065 5/438	0.252 ± 0.076 5/438	0.564 ± 0.076 5/438	0.002 ± 0.002 5/438	19 ± 7 (10–35)

^a 5 mM D-glutamate/5 mM D-aspartate (GLU/ASP)

^b parameters are for the “best site” in each brain locomotor area (see Methods)

^c parameters for muscle burst activity during swimming from Davis et al., 1993 and Paggett et al., 1998

^d number of animals/number of cycles

^e range of latencies for initiated locomotor activity

* - P ≤ 0.01;

** - P ≤ 0.001; significantly different than cycle times for locomotor activity in whole animals (ANOVA, Bonferroni multiple comparisons post-test)

TABLE 2

INDIVIDUAL AND AVERAGE SIZES OF BRAIN LOCOMOTOR AREAS^a

Locomotor Area	Experiment Number	Width ^b (μm)	Length ^c (μm)	Cycle Time ^d (ms)
VMD (n = 5)	AJ 239	92	376	788 ± 180 (98)
	AJ 212	90	400	1032 ± 97 (98)
	AJ 209	375	120	1113 ± 110 (86)
	AJ 210	60	125	1211 ± 235 (99)
	AJ 208	50	50	1741 ± 348 (36)
	MEAN	133 ± 136	214 ± 162	
DLM (n = 5)	AJ 240	420	100	1252 ± 675 (98)
	AJ 212	150	200	1288 ± 227 (85)
	AJ 207	265	110	1321 ± 138 (41)
	AJ 208	165	70	1701 ± 229 (33)
	AJ 213	220	125	2013 ± 397 (65)
	MEAN	244 ± 109	134 ± 49	
RLR (n = 5)	AJ 212	100	190	877 ± 145 (83)
	AJ 249	70	300	980 ± 212 (65)
	AJ 211	375	400	998 ± 154 (99)
	AJ 252	110	60	1101 ± 241 (98)
	AJ 250	65	190	1568 ± 400 (93)
	MEAN	144 ± 131	228 ± 128	

^a pharmacological stimulation with 5 mM D-glutamate/5 mM D-aspartate (GLU/ASP)

^b maximum extent along the transverse axis (see Methods)

^c maximum extent along the rostrocaudal axis

^d cycle times are for the “best site” in each brain locomotor area (see Methods); number of cycles in parentheses

Note that brain locomotor areas are not rectangular (see Figs. 2A,B and 4A,B), and the full area of a locomotor region was less than width * length

TABLE 3
PARAMETERS OF LOCOMOTOR ACTIVITY INITIATED BY DIFFERENT PHARMACOLOGICAL AGENTS IN BRAIN LOCOMOTOR AREAS^a

Locomotor Area	T (ms)	BPProstral	BPcaudal	Φ_{RT-LT}	Φ_{INT}	Latency (sec.)
VMD						
GLU/ASP	958 ± 469 3/288 ^b	0.346 ± 0.115 3/288	0.353 ± 0.149 3/288	0.532 ± 0.179 3/288	0.003 ± 0.003 3/288	12 ± 4 (4–18) ^c
NMDA	930 ± 191 3/183	0.337 ± 0.045 3/183	0.356 ± 0.069 3/183	0.495 ± 0.080 3/183	0.003 ± 0.002 3/183	27 ± 11 (11–44)
AMPA	1053 ± 367 3/132	0.348 ± 0.053 3/132	0.390 ± 0.101 3/132	0.458 ± 0.092 3/132	0.005 ± 0.003 3/132	11 ± 12 (3–35)
KA	957 ± 358 3/155	0.346 ± 0.056 3/155	0.304 ± 0.120 3/155	0.566 ± 0.087 3/155	0.005 ± 0.003 3/155	13 ± 9 (2–26)
DLM						
GLU/ASP	893 ± 319 3/297	0.362 ± 0.053 3/297	0.304 ± 0.080 3/297	0.579 ± 0.080 3/297	0.002 ± 0.003 3/297	21 ± 14 (4–45)
NMDA	817 ± 256 3/240	0.362 ± 0.061 3/240	0.297 ± 0.060 3/240	0.617 ± 0.071 3/240	0.003 ± 0.003 3/240	32 ± 15 (11–52)
AMPA	907 ± 374 3/236	0.342 ± 0.057 3/236	0.292 ± 0.062 3/236	0.590 ± 0.074 3/236	0.002 ± 0.003 3/236	13 ± 18 (2–54)
KA	968 ± 359 3/138	0.350 ± 0.056 3/138	0.285 ± 0.062 3/138	0.581 ± 0.081 3/138	0.003 ± 0.003 3/138	21 ± 19 (2–51)
RLR						
GLU/ASP	1051 ± 374 8/449	0.349 ± 0.069 8/449	0.333 ± 0.093 8/449	0.496 ± 0.078 8/449	0.005 ± 0.003 8/449	16 ± 9 (5–40)
NMDA	1246 ± 476 6/282	0.388 ± 0.070 6/282	0.358 ± 0.107 6/282	0.520 ± 0.087 6/282	0.005 ± 0.003 6/282	30 ± 12 (16–50)
AMPA	1466 ± 572 4/73	0.313 ± 0.077 4/73	0.296 ± 0.128 4/73	0.484 ± 0.127 4/73	0.005 ± 0.003 4/73	13 ± 15 (2–45)
KA	1131 ± 246 2/92	0.335 ± 0.065 2/92	0.298 ± 0.060 2/92	0.529 ± 0.070 2/92	0.005 ± 0.002 2/92	5 ± 2 (3–7)

^a parameters are for the “best site” in each brain locomotor area (see Methods)

^b number of animals/number of cycles; note that for stimulation in the VMD or DLM locomotor areas, the same three animals were used to test all of the pharmacological agents

^c range of latencies for initiated locomotor activity

Statistics: ANOVA, Bonferroni multiple comparisons post-test (values for a given locomotor parameter were not significantly different for different locomotor areas and agents)