Diencephalic Locomotor Region in the Lamprey—Afferents and Efferent Control

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Ménard A, Grillner S. Diencephalic locomotor region in the lamprey—afferents and efferent control. J Neurophysiol 100: 1343–1353, 2008. First published July 2, 2008; doi:10.1152/jn.01128.2007. In vertebrates, locomotion can be initiated by stimulation of the diencephalic locomotor region (DLR). Little is known of the different forebrain regions that provide input to the neurons in DLR. In the lamprey, it had been shown previously that DLR provides monosynaptic input to reticulospinal neurons, which in turn elicit rhythmic ventral root activity at the spinal level. To show that actual locomotor movements are produced from DLR, we use a semi-intact preparation in which the brain stem is exposed and the head fixed, while the body is left to generate actual swimming movements. DLR stimulation induced symmetric locomotor movements with an undulatory wave transmitted along the body. To explore if DLR is under tonic GABAergic input under resting conditions, as in mammals, GABAergic antagonists and agonists were locally administered into DLR. Injections of GABA agonists inhibited locomotion, whereas GABA antagonists facilitated the induction of locomotion. These findings suggest that GABAergic projections provide tonic inhibition that once turned off can release locomotion. Double-labeling experiments were carried out to identify GABAergic projections to the DLR. Populations of GABAergic projection neurons to DLR originated in the caudoventral striatal area. These different GABAergic projection neurons, which also project to other brain stem motor centers, may represent the basal ganglia output to DLR. Moreover, electrical stimulation of striatum induced long-lasting plateau potentials in reticulospinal cells and associated locomotor episodes dependent on DLR being intact, suggesting that striatum may act via the basal ganglia output identified here.

INTRODUCTION

Having emerged in vertebrate evolution ~560 million years ago (Kumar and Hedges 1998), lampreys represent the most ancient vertebrate group that has developed all main parts of the CNS. Because of their critical position in vertebrate evolution and their relative simplicity, they have been used as an experimental model system for the analyses of the cellular bases of vertebrate motor behavior. The lamprey brain stem spinal cord circuitry responsible for locomotion, posture, and steering has been described in considerable detail (for review, see Grillner et al. 2000) and provides now a solid platform to address questions concerning the supraspinal control of locomotion.

In lampreys, as in other vertebrates, locomotor activity can be initiated by stimulation of the di- or mesencephalic locomotor regions (DLR, MLR). These regions contain neurons that project monosynaptically to reticulospinal neurons that in turn activate the spinal locomotor networks (Brocard and Dubuc 2003; El Manira et al. 1997; Le Ray et al. 2003). The MLR has been investigated in many classes of vertebrates including the lamprey and can be described as a command center that can initiate and control the frequency of locomotor movements (Brudzynski et al. 1986; Garcia-Rill et al. 1985; Shik et al. 1966; Sirota et al. 2000; for review, see Grillner et al. 1997; Jordan 1998). The DLR, on the other hand, has received less attention. It corresponds to the area originally (but incorrectly) designated as the subthalamic locomotor region in cats (Grossman 1958; Orlovsky 1970; see Grillner et al. 1997; Jordan 1998) and the lateral hypothalamus in rats (Sinnamon 1993 see also Milner and Mogenson 1988).

The lamprey DLR coincides with a region referred to as the ventral thalamus (El Manira et al. 1997; see also Pombal and Puelles 1999), which contains neurons that project to the lower brain stem reticulospinal nuclei. Moreover, in in vitro brain/spinal cord preparations, electrical stimulation of this region elicits monosynaptic excitatory postsynaptic potentials (EPSPs) and rhythmic firing in reticulospinal neurons as well as rhythmic locomotor-like activity in the ventral roots (El Manira et al. 1997). In the present study, one aim was to investigate in the semi-intact preparation (Sirota et al. 2000) if the rhythmic activity generated by stimulation of the DLR actually produced well-coordinated locomotor movements and if the level of activity could also be controlled from DLR. The semi-intact preparation leaves the body free to move, while the brain stem is exposed but held immobile.

A second goal was to describe the afferent input to DLR, in particular in relation to the input from GABAergic neuronal structures. We have recently shown that both MLR and tectum (controlling eye and orientation movements) both receive GABAergic projections from certain pallial regions (Ménard et al. 2007; Robertson et al. 2006). These areas in turn receive GABAergic projections from the lamprey striatum, and they may therefore correspond to the output nuclei of the lamprey basal ganglia. The striatum of the lamprey has a similar organization to that of mammals with regard to neural elements and afferent input (Pombal and Puelles 1999). A dopamine depolarization produces severe hypokinetic deficits of the same type as in mammals (Ménard et al. 2003, 2004; Grillner et al. 2000; Thompson et al. 2008). In mammals, GABAergic cells from the output nuclei of the basal ganglia control different motor centers, including tectum/superior colliculus and MLR through tonic inhibition at rest (Garcia-Rill 1986; Hikosaka et al. 2000; Mink 1996; Takakusaki et al. 2003; see Grillner et al. 2005).
The same motor centers receive tonic GABAergic inhibition in lamprey (Ménard et al. 2007; Robertson et al. 2006). In the present study, we therefore investigate the GABAergic modulation of the lamprey DLR and the forebrain GABAergic projections to the DLR.

METHODS

All experiments were conducted in compliance with the regulations for the care and use of laboratory animals of the local ethical committee (Stockholm Norra Djurförsöksstäts Nämnd) and the Swedish animal protection society (Djurskydds Myndigheten).

Physiology

Seventy-five postmetamorphic adult lampreys 37 Petromyzon marinus (length 10–15 cm), 38 Lampetra fluviatilis (length around 30 cm) were used for the physiological experiments. No obvious differences have been found between the two lamprey species with regard to both physiology of the motor system (see Grillner et al. 2008) and the distribution of GABAergic neurons (Robertson et al. 2007) and other transmitter-identified systems.

Surgical procedures

During the surgery, the animals were deeply anesthetized with tricaine methane sulfonate (MS-222; 100 mg/l; Sigma, St. Louis, MO) diluted in fresh water. In semi-intact preparations, the brain was exposed dorsally by removing all the muscles, and connective tissue from the head region to the fourth gill while the rest of the body was kept intact. To discontinue anesthesia, a rostral portion of the telencephalon was removed (the olfactory bulbs and the dorsal half of the lateral pallium). The habenular and posterior commissures as well as the tectum were cut open dorsally to allow access to the DLR. The animals were then transferred to a chamber perfused with cooled (8–12°C) Ringer (which contained, in mM: 138 NaCl, 2.1 KCl, 1.8 CaCl2, 1.2 MgCl2, 4 glucose, and 2 HEPES; pH of 7.4) in which the isolated brain was pinned down but the tail free to move (Fig. 1A). In in vitro preparations, the brain and the rostral spinal cord were dissected out of the animal and pinned down in a chamber perfused with cooled Ringer.

Recordings

In semi-intact preparations, locomotion was monitored with a video camera (Panasonic NV-GS 11, Tokyo, Japan; sampling rate: 25 frame/s) and electromyograms (EMGs). EMGs of the myotomes caudal to the dorsal fin on each side of the body of the lamprey were recorded with pairs of single stranded Teflon-insulated stainless steel wires (0.5 mm ID, 1.125 mm OD; A-M Systems, Carlsborg, WA). The signals were differentially amplified (Differential AC amplifier No. 1700, A-M Systems; 10,000×), filtered (bandwidth: 100 Hz to 5 kHz) and acquired at a sampling rate of 10 kHz through a Digidata 1200 interface (Axon Instruments, Union City, CA) with Clampex 9 software (Axon Instruments). Video frames were captured in AVI format with the software Pinnacle Hollywood FX 8, and the outlines of the body of the lamprey were drawn in Flash MX (Macromedia, Toronto, ON, Canada). In in vitro preparations, fictive locomotion was monitored with electromyograms (EMGs). EMGs were recorded from the ventral roots with glass suction electrodes filled with Ringer solution and band-pass filtered between 100 and 1,000 Hz. Intracellular recordings of reticulospinal cells were performed with sharp glass micropipettes pulled from thin-walled aluminosilicate glass (Sutter Instrument, Novato, CA) to a final resistance of 80–120 MΩ, filled with 3 M potassium acetate. The intracellular signals were recorded with an Axoclamp 2A amplifier (Axon Instruments) and amplified 50 times. EMGs and ENGs were analyzed with Datapac software (Run Technologies, Mission Viejo, CA).

FIG. 1. Analysis of the swimming induced by stimulation of diencephalic locomotor region (DLR) in semi-intact preparations. A: schematic representation of the semi-intact preparation showing the expose brain to the left and body right and the position of the electrodes. B: photomicrograph of a cross-section through the DLR (see also Fig. 5), indicating where the microelectrode was positioned and where drugs and tracer were injected. Scale bar = 200 μm. C: electromyographic (EMG) recordings acquired during a locomotor activity evoked by a continuous electrical stimulation of the DLR (0.5 Hz 15 μA) that shows clear left and right alternation of the muscle fibers. D: comparison of the swimming movements induced by electrical stimulation of the DLR (10 pulses at 10 Hz, 20 μA; D1) and by injection of n-glutamate (5 mM; D2) into the DLR of the same animal. The outlines of the body of the lamprey were drawn for each video frame within 1 locomotor cycle. E1–E3: graphs showing the effect of changes of the intensity of a continuous stimulation (0.5 Hz) on the frequency of swimming (E1), the locomotor burst duration (E2), and the locomotor burst mean amplitude (E3) of the same animal as in D.
Stimulations and drug injections

The habenula, the fasciculus retroflexus, the infundibular recess, and the ventriculus lateralis were used as landmarks to precisely position the stimulating electrode into the DLR (El Manira et al. 1997). The medial pallium, the ventriculus lateralis, and the nucleus preopticus were used as markers to identify the position of the striatum. The DLR was also identified physiologically as the best location in the thalamus in which locomotion could be induced by unilateral electrical or chemical stimulation. The size of the effective region was ~100–300 μm in diameter. Injection of a fluorescent tracer into the stimulation site at the end of the experiment was done for six animals to confirm the position of the electrode determined by inspecting 20 μm transversal sections of the brain (Fig. 1B). Electrical stimulation was produced with an isolated pulse stimulator No. 2100 (A-M Systems) connected to tungsten concentric bipolar electrodes (SNEX-100, 100 μm cross-section, impedance: 0.7–1.5 MΩ, epoxy-lute insulation; Clark Electromedical Instruments, Pangbourne, UK). Chemical stimulation was produced with α-glutamate (5 mM; Sigma) pressure-injected with a Picospritzer II (Parker Hannifin, General Valve Division, Fairfield, NJ) through a glass micropipette lowered into the tissue just below the surface. Ten- to 20-ms pulses at 20 psi were used to inject gabazine hydrobromide (SR 95531, 1 mM; Tocris, Ellisville, MO), or muscimol hydrobromide (1 mM; Sigma) into the brain. All drugs were diluted in Ringer’s containing Fast Green to visualize the extent of the injection.

RESULTS

DLR stimulation induced locomotion in the semi-intact preparation

The movements that result from DLR activation were investigated in 25 semi-intact preparations, which allow an analysis of the overall coordination along the entire body (Fig. 1A). Electrical stimulation of the DLR evoked rhythmic, symmetric, and well-coordinated swimming movements in 12 of 17 animals. The movements were similar to those of intact animals, which can be described as a wave of lateral displacement of the lamprey body that propagates from head to tail which is symmetric on the right and left side of the body and is of larger amplitude toward the tail (Fig. 1D) (Williams et al. 1989). DLR stimulation did not induced disorganized patterns of motor activity such as C or S shapes (Sirota et al. 2000).

The EMG activity alternated between the left and right side of the body (Fig. 1C). The quality of swimming evoked by stimulation of the DLR was comparable to that induced from MLR in the same animal (n = 10; data not shown). In the cases in which the electrical stimulation failed to induce locomotion, the electrode was moved to other nearby positions within the DLR area delimited by our specific landmarks. In five animals, this was not sufficient to induce swimming, and in some of these animals, a disorganized pattern of movement was instead produced.

To exclude the possibility that the electrical stimulation was producing its effect through activation of fibers of passage, we also stimulated the DLR by a microinjection of α-glutamate that would excite only the cell bodies. In four animals, the swimming movements induced by an electrical stimulation of the DLR (10 pulses, 10 Hz, 20 μA, Fig. 1D1) were compared with those induced by a glutamate injection (5 mM, Fig. 1D2). The swimming movements were qualitatively similar as was the frequency (2.3 vs. 2.1 Hz) and the rostrocaudal phase lag from head to tailfin. The lateral displacement was somewhat larger following electrical stimulation. This indicates that neurons within the DLR can induce swimming.

The three graphs in Fig. 1E illustrate how the EMG locomotor burst activity changes with the amplitude of the DLR stimulation (from 7 to 20 μA) during continuous train of stimulation (0.5 Hz). The frequency of swimming increased with the strength of the electrical stimulation of the DLR (Fig. 1E1). Burst amplitude (Fig. 1E3) increased with current intensity, while the burst duration (Fig. 1E2) became correspondingly shorter. This is most likely due to both a recruitment of

Anatomy

SURGICAL PROCEDURES USED FOR TRACER INJECTIONS. Anatomical experiments were performed on 22 adult lampreys (Lampetra fluviatilis). The animals were kept in a bath perfused with Ringer solution at 8–12°C containing the anesthetic MS222 throughout the procedure and were spinalized at the most rostral level of the spinal cord. The brain was exposed by a minimal opening of the skull and the habenular and posterior commissures as well as the tectum were cut open dorsally to allow access to the DLR. Neurobiotin (Vector, Burlingame, CA; 10–15% in distilled water containing fast green) was injected into the DLR with a glass micropipette connected to a Picospritzer. The landmarks described in the preceding text were used to precisely position the glass micropipette into the DLR. The animals were then left to rest in the bath for 24 h to allow transport of the tracer after which they were bath for 24 h to allow transport of the tracer after which they were

GABA immunohistochemistry and tracer visualization

Sections were preincubated with 1% bovine serum albumin (BSA), 0.3% Triton X-100 in 0.1 M PB for 1 h. The primary antibody, a mouse monoclonal anti-GABA (0.1 μg/ml), was then applied to the sections for 1 day at 4°C. After thorough rinsing in 0.01 M PB saline (PBS), the sections were incubated for 1 h in a mixture of streptavidin conjugated to Cy3 (1:1,000; Jackson Immunoresearch, West Grove, PA) or to Alexa 488 (1:1,000; Molecular Probes, Eugene, OR) for visualization of the Neurobiotin and donkey anti-mouse IgG conjugated to Cy3 (1:800; Jackson Immunoresearch) for visualization of GABA antibodies. Sections were finally thoroughly rinsed in PBS and mounted in glycerol containing 2.5% diazabicyclooctanate (Sigma).

The monoclonal anti-GABA antibody mAb 3A12 was kindly donated by Dr. Peter Streit, Zürich, Switzerland. This antibody was developed after immunization with GABA coupled to BSA and has been well characterized by enzyme-linked immunosorbent assays and preabsorption with GABA-BSA (Matute and Streit 1986). Colo- localization with mAb 3A12 and a rabbit polyclonal anti-GABA antibody (1:100; No. AB131; Chemicon, Temecula, CA) showed immu

noreactivity in the same cells in the lamprey brain. No immunoreac- tivity was detected when the primary antibody was omitted, and the sections were incubated with only the secondary antibody.

Analysis

Only the brains showing an injection site into the ventral thalamus, rostral to the fasciculus retroflexus, caudal to the nucleus of the postoptic commissure and dorsal to the hypothalamus and a good GABA labeling were included in the analysis (n = 9). Sections from the olfactory bulb caudally to the injection site were analyzed on a Nikon fluorescence microscope, and photomicrographs were taken with a Zeiss AxioCam digital camera (Carl Zeiss AB, Stockholm). Illustrations were prepared in Adobe Photoshop CS1. The nomenclature used is adapted from previous studies (Heier 1948; Nieuwenhuys and Nicholson 1998; Pombal and Puelles 1999; Schober 1964).
new motoneurons (previously subthreshold) and an enhanced frequency of already active motoneurons.

**DLR stimulation induced fictive locomotion in the in vitro preparation**

We analyzed in greater detail the characteristics of the fictive locomotion induced by DLR stimulation in the in vitro preparation (13 of 22 preparations investigated), which is exempt of sensory feedback.

As illustrated in Fig. 2A1, a train of 10 pulses (10 Hz, 50 μA) could induce a bout of locomotor activity that lasted for several seconds. The longer the stimulus train, the longer the locomotor episode (not illustrated). When a continuous train of stimulation (0.5 Hz, 50 μA) was instead delivered to the DLR, the locomotor episode was maintained for a much longer period (Fig. 2B1; n = 4). Moreover, the continuous train increased the locomotion rate as can be assessed by the comparison of the magnified ENGs in Fig. 2A2 and B2. The graphs of the instantaneous locomotor frequency obtained in each situation show that the rate decreased progressively during a locomotor episode induced by a short train of pulses but that it remained stable at a higher level during a locomotor episode induced by a continuous train of stimulation (Fig. 2C).

As in the semi-intact preparations, the rate of locomotor activity can be modulated by the parameters of the continuous train of stimulation. Figure 2D provides an example of the relationship between the frequency of stimulation and the rate of locomotion that increased with higher stimulus frequencies.

It is noteworthy that even a stimulation given as seldom as every 10 s can induce a locomotor episode.

**GABAergic input to the DLR modulates swimming in lampreys**

The significance of the GABAergic input to the DLR was tested in 10 in vitro and 14 semi-intact preparations by injecting a GABA_A agonist (muscimol) and an antagonist (gabazine) into the DLR while monitoring locomotor activity.

For the effect of the GABA_A agonist, a control locomotor episode was induced by the injection of α-glutamate (5 mM; Fig. 3A). The EMG recordings show that swimming occurred 4 s after the onset of the injection and lasted for 14 s. Figure 3B shows the effect of muscimol when injected during a α-glutamate evoked locomotor bout and 38 s later in Fig. 3C where the α-glutamate response had been entirely abolished. However, once the drug was washed out after 15 min, α-glutamate regained its efficacy (Fig. 3D). The results obtained in each animal tested (n = 7) were qualitatively the same. The experiments thus show that cells within DLR have GABA_A receptors and that when activated they can modulate locomotor activity.

To test whether there is a tonic release of GABA at the level of the DLR at rest, a GABA_A antagonist (gabazine; 1 mM) was injected into the DLR. In only 1 of 17 animals did this evoke locomotor activity. However, gabazine facilitated the initiation of locomotion following injection of α-glutamate into the DLR in 12 animals. In the first example (Fig. 4, A1–C1), locomotion was induced by α-glutamate in the control (Fig. 4A1), but after gabazine itself (Fig. 4B1) no activity was elicited, the locomo-
The effect of muscimol injection into the DLR during swimming. A: EMG recordings in which a control bout of swimming was induced by an injection of D-glutamate (5 mM) into the DLR (▲). B: the swimming evoked by D-glutamate (2st ▲) was reduced in intensity and prematurely stopped when muscimol (1 mM) was injected into the DLR (3rd ▲). C: 38 s following the injection of muscimol in which swimming could not be initiated by D-glutamate. D: once the muscimol had washed out, swimming was again induced by an injection of D-glutamate into the DLR.

The effect of gabazine injection into the DLR. A1 and A2: EMGs in the control condition in which an injection of D-glutamate (5 mM) into the DLR of 2 different lampreys evoked locomotion in 1 animal (A1) or was subthreshold for locomotion in another (A2). B1 and B2: injection of gabazine (1 mM) alone did not induce locomotor activity in EMGs. C1 and C2: test condition around 30 s following the injection of gabazine where D-glutamate initiated swimming that had greater intensity than that with D-glutamate alone (C1) or where the subthreshold D-glutamate injection then evoked locomotion (C2). D1 and D2: graphs comparing the instantaneous frequency (D1) and the burst amplitude (D2) of locomotion induced in control (▼) and in the presence of gabazine (●).
was not induced (Fig. 4A2). Neither did gabazine alone induce activity (Fig. 4B2). But, in the test condition, in which gabazine was still present in the DLR, subsequent injections of \( \text{d}-\text{glutamate} \) were not induced (Fig. 4A2). Neither did gabazine alone induce activity (Fig. 4B2). But, in the test condition, in which gabazine was still present in the DLR, subsequent injections of \( \text{d}-\text{glutamate} \) were not induced (Fig. 4A2). Neither did gabazine alone induce activity (Fig. 4B2). But, in the test condition, in which gabazine was still present in the DLR, subsequent injections of \( \text{d}-\text{glutamate} \) were not induced (Fig. 4A2).

Forebrain GABAergic projections to the DLR

To investigate if some of the projecting neurons to the DLR were GABAergic, we combined retrograde tracing, after injection of Neurobiotin into DRL, with GABA immunohistochemistry (9 animals). The distribution of Neurobiotin-labeled cells and tracts were in accordance with previous studies (El Manira et al. 1997; see also Northcutt and Wicht 1997). Figure 5 shows a schematic representation of the distribution. Retrogradely labeled tracts are depicted in yellow and retrogradely labeled cells in green on the left side of the brain sections. From the injection site, some fibers descend toward the hypothalamus, others decussate in the postoptic commissure to form a contralateral bundle of fibers (Fig. 6A), but the majority of the labeled fibers ascend rostrally close to the optic tract (Figs. 5D and 6A). At the transition between the diencephalon and the telencephalon, the fibers bifurcate laterally toward the lateral pallium. Some very fine fibers course through the medial pallium extending to the septum (Fig. 5, C–E). Many cells were located bilaterally in the thalamus, the hypothalamus, the nucleus of the postoptic commissure (NCPO), and the preoptic nucleus (PO; Fig. 5, C–E). Others were found mainly ipsilaterally in the pallial and striatal areas. In most animals, a group of cells were labeled in the caudalventral portion of the medial pallium and others in its periventricular cell layer (Figs. 5D and 6B). A more scattered distribution of retrogradely labeled neurons was observed in the lateral pallium extending to the septum (Fig. 5, B and C). Many cells were concentrated in the area shared with the dorsal pallium and the lateral portion of the striatum and surrounding area (Fig. 5C).

Except for the PO, all areas with retrogradely labeled cells were found to contain GABAergic cells (see right side of the brain sections). However, the number of double labeled cells was limited. In the caudoventral portion of the medial pallium, lateral to the ementia thalami, a discrete group of GABAergic cells (Fig. 6B) is located, in which some cells were double labeled (Fig. 5D). Figure 6D shows an example of such a double-labeled cell found among other single labeled neurons. Double-labeled cells were also found in the dorsal and ventral portion of the lateral pallium (Fig. 5C). Figure 6E illustrates another double-labeled cell located in the ventral portion of the lateral pallium. Finally, several double-labeled cells were found in the region of the dorsal pallium (Figs. 5C and 6E) and the lateral portion of the striatum (Figs. 5C and 6F). In the NCPO and hypothalamus, where an abundant number of retrogradely labeled cells were found and where GABAergic cells are densely packed, almost no double-labeled cells were observed. As illustrated in Fig. 6C, these cells form distinct subpopulations, the GABAergic neurons being smaller and rounder in shape than the non-GABAergic retrogradely labeled cells.

Striatal contribution to locomotion via DLR

If the basal ganglia output is at the origin of the GABAergic modulation of the DLR, with stimulation of the striatum, the output level of the basal ganglia may be expected to be inhibited and thereby disinhibit DLR and facilitate locomotion. To monitor the effects of the striatal stimulation, we recorded intracellularly from reticulospinal cells in rhombencephalon and the locomotor activity through EMG recordings. Electrical stimulation of the striatum (see METHODS) induced locomotion in 8 of 15 preparations. Figure 7A, inset, shows that the electric pulse induced a polysynaptic EPSP in the reticulospinal cell. A continuous train of electric stimulation (1 Hz, 40 \( \mu \)A), induced after a few seconds a long plateau of depolarization and action potentials (Fig. 7A). Around 10 s after the rising phase of the plateau, a locomotor episode started (Fig. 7B). The locomotion outlasted the electrical stimulation and continued for the

**FIG. 5.** Schematic representation of the distribution of GABA, retrogradely and double-labeled cells in the forebrain of the lamprey. A: drawing of the dorsal surface of the brain of *Lamproptera fluviatilis* where the levels of the transverse sections (B–E) and the injection site (green dot and large gray circle in B) are indicated. B–E: standardized schematics drawings of transverse sections from rostral (B) to caudal (E) levels. GABA-immunoreactive cells are represented as red dots on the right side of the sections, and retrogradely labeled cells and fibers are in green and yellow, respectively on the left side. Double labeled cells are represented as green dots circled with red on the left side. Note the presence of double-labeled neurons in the caudal part of the medial pallium (MPal; D), and in the striatum, dorsal pallium and lateral pallium (Str, DPal, LPal; C). Scale bar: 300 \( \mu \)m.
whole duration of the plateau in the reticulospinal cell (n = 4/6). A direct correlation between the plateau depolarization in single reticulospinal cells and the locomotor activity was not clear-cut (Fig. 7B2), although the firing rate of the cells increased from the beginning to the middle of the plateau (4–10 Hz) as did the amplitude of the bursts.

To investigate if DLR contributed to the locomotor response induced by stimulation of the striatum, the basic level of activity in the DLR was modified as we monitored the effect on the locomotor activity. In Fig. 8A, the striatum was stimulated below threshold for evoking a reticulospinal plateau potential or motor activity. Without striatal stimulation, d-glutamate was then injected into the DLR to induce a small plateau potential (8 s long) in the reticulospinal cell and a short bout of motor activity in the EMGs but not robust locomotion (Fig. 8B). When these two stimulations were combined, a prominent, long-lasting plateau potential (14 times longer than under control conditions) was induced accompanied by a locomotor episode (Fig. 8C). To test the effect of DLR inhibition, the striatum was stimulated with electric trains (10 pulses, 20 Hz, 50 μA) to induce a plateau potential in the reticulospinal cell and locomotion (Fig. 8D). After injection of muscimol (1 mM) into the DLR, an identical stimulation of the striatum neither produced a plateau potential nor locomotor activity (Fig. 8E). Each stimulus pulse of the striatum still evoked a polysynaptic EPSP in the reticulospinal cell, but it was of smaller amplitude after muscimol injection into the DLR (gray trace in Fig. 8F).

**DISCUSSION**

**Characterization of the DLR command**

It was previously reported that stimulation of the DLR evoked rhythmic alternating ventral root activity in in vitro preparations exempt of sensory feedback (El Manira et al. 1997). In the present study, we demonstrate that the movements resulting from DLR activation represent swimming that is similar to that of the intact animal. Because in the present study active swimming was also induced by γ-glutamate in the DLR, we conclude that neurons within DLR, rather than fibers of passage, are responsible for the initiation by electrical stimulation. Moreover, we show that DLR could control the rate of locomotion and the level of the muscle output.

The DLR thus fulfills the criteria required to be described as a locomotor command center. Thus together with MLR (Sirota et al. 2000), it is established that there are two locomotor command centers present in the lamprey as in other vertebrates. Each of them has monosynaptic excitatory connections to reticulospinal neurons in the middle and posterior rhombencephalic reticular nuclei (El Manira et al. 1997; Sirota et al. 2000), which conveys excitation to the spinal locomotor networks. Each can thus elicit locomotion independently, depending perhaps on the behavioral context. Whether there is a direct interaction between MLR and DLR is not yet known.

**GABAergic input to the DLR modulates swimming**

In the physiological experiments, GABA_A antagonists and agonists were injected into the DLR to assess a possible role for the GABAergic inputs. Whereas GABA_A agonists blocked locomotion consistently, GABA_A antagonists only occasionally induced locomotion when injected into the DLR. However, gabazine facilitated the induction of locomotion with γ-glutamate injections into the DLR and increased EMG amplitudes of ongoing activity. These results with both muscimol and gabazine indicate not only that neurons within DLR have GABA_A receptors but also that a blockade of GABA_A receptors can facilitate the occurrence of a locomotor response induced by stimulation of DLR and receptor activation can depress it.

A similar study with gabazine on MLR concluded that the GABAergic projections provide a tonic inhibition that once turned off will release the locomotor command (Ménard et al. 2007). The GABAergic input to the DLR, although present, thus appears to be weaker which would explain why the disinhibition produced by gabazine was not sufficient to induce locomotor activity. However, when extra excitation was provided, the presence of a tonic inhibition could be disclosed.
Forebrain GABAergic projections to the DLR

In a previous study, it was shown that neurons in the lateral pallium and other regions project to the DLR (El Manira et al. 1997). In a detailed anatomical report, we recently showed that the neurotransmitter GABA is widely distributed in the adult lamprey brain (Robertson et al. 2007). To identify the GABAergic projections from the forebrain to the DLR, we used the double-labeling technique.

A limited number of double-labeled cells were found in different regions of the forebrain. The available data do not allow a quantitative comparison of the descending GABAergic projections to MLR (Ménard et al. 2007) and DLR, respectively. The finding that the effect of the GABA_A receptor antagonist on DLR was less prominent than on MLR could depend on the relative density of GABA projections but also to a variety of other factors. The regions of the forebrain, in which double-labeled GABAergic projection neurons to MLR and DLR were found, are overlapping. They include the medial, dorsal, and lateral pallium.

In anurans, striatal neurons project to an area located just caudal to striatum that is considered to represent the dorsal pallidum (Endepols et al. 2004; Muhlenbrock–Lenter et al. 2005). This area projects to the dorsal thalamus. In amniotes, GABAergic neurons serve as interneurons within pallium and not as output neurons with long axons. We therefore explore the possibility that the long GABAergic projections from specific portions of the pallium to the DLR, identified here, may correspond to the output nuclei of the basal ganglia.

Lateral to the ementia thalami, in the ventrocaudal part of the medial pallium, a discrete group of GABAergic cells were double-labeled. GABAergic projections to the tectum and the MLR also originate from the same area (Ménard et al. 2007; Robertson et al. 2006). This area is located just caudal to striatum, similar to the condition in anurans. In teleosts, the ementia thalami is thought to be derived from the diencephalon and a region located more laterally is referred to as the entopeduncular complex and is considered to be of telencephalic origin (Braford and Northcutt 1983; Wullimann and Mueller 2004a; Wullimann et al. 1996). The mammalian entopeduncular nucleus is an integral part of the pallidum of the basal ganglia. The anuran anterior entopeduncular nucleus is now homologized with part of the mammalian pallidum but with the teleostean entopeduncular complex the situation is
unclear (Wullimann and Mueller 2004b). The homology with the caudoventral portion of the medial pallium in lamprey, and the entopeduncular complex is as yet to be confirmed. It contains, however, GABAergic projecting neurons to the same brain stem structures as in mammals. Projections from striatum to this area have been established (Northcutt and Wicht 1997; Pombal et al. 1997b; Robertson et al. 2007).

Based on neurohistochemical criteria, the GABAergic neu-
rons in the ventral part of the lateral pallium in the lamprey have been proposed to be homologous to the ventral pallidum as described in anamniotes and amniotes (Pombal et al. 1997a; Reiner et al. 1998; Weigle and Northcutt 1999). In the present study, this region was found to have GABAergic projecting neurons to DLR and previously also to MLR (Ménard et al. 2007). The present study also demonstrated a direct GABAergic projection from the area of striatum as previously for MLR (Ménard et al. 2007). Whether striatal GABAergic cells provide direct inhibition to the DLR or if this region belongs to a subpopulation of pallidal neurons that would intermingle with striatal neurons is as yet unknown.

Because the gene Nkx2.1, considered to be important for the development of the pallidum (Sussel et al. 1999), is not expressed in the ventral telencephalon of lamprey embryos (Murakami et al. 2005; Osorio et al. 2005), the presence of a pallidum in lamprey has been questioned. These studies are, however, limited to a few embryonic stages. The input layer of the basal ganglia, the striatum, has qualities that are found in higher vertebrates, which suggests that the general functional organization of the basal ganglia may be similar to that of mammals. The lamprey striatum thus contains spiny neurons immunoreactive to GABA, substance P, enkephalin, and neurons positive for acetylcholinesterase (Pombal et al. 1997a). Patch-clamp recordings show that striatal neurons in lamprey express the hallmarks of mammalian striatal neurons such as inward rectifier and I_H channels (Ericsson et al. 2007). The striatum receives input from a variety of structures (thalamus, pallium), including a strong dopaminergic, serotonergic, and histaminergic input from neurons of the same origin as in mammals (Pombal et al. 1997a,b). The striatum also sends axons to medial pallium (Northcutt and Wicht 1997), and the lateral pallium (Pombal et al. 1997a). Dopamine depletion results in the same type of hypokinetic symptoms as in mammals, which are counteracted by administration of the dopamine agonist apomorphine (Thompson et al. 2008).

In addition to the double-labeled GABAergic projection neurons, there are a number of other areas which are retrogradely labeled from DLR (El Manira et al. 1997), which have also been identified here. They may represent input to DLR, but the possibility that some fibers of passage have been labeled must also be considered. Even though our injections were aimed at the periventricular cell layer of the ventral thalamus, some axons may have been interrupted en route to their final target. Many fibers in the medial pallium were labeled, and it is considered a main telencephalic target of the thalamus (Northcutt and Wicht 1997; Polenova and Vesselkin 1993). Mitral cells of the medial olfactory bulb were retrogradely labeled (El Manira et al. 1997). Their axons course medially through the thalamus to continue to the caudal hypothalamus (Northcutt and Puzdrowski 1988; Northcutt and Wicht 1997). Other descending tracts originating from different pallial areas to the habenulae, pretectum, tectum, or midbrain could also have been labeled by the injection (Northcutt and Wicht 1997; Polenova and Vesselkin 1993; for review, see Nieuwenhuys and Nicholson 1998).

**DLR relation to the striatum**

Given that both the structure and function of the striatum appear to be conserved from cyclostomes to mammals, it would appear likely that striatum acts via the putative pallidal structures with GABAergic projection neurons that target the DLR. In the present study, electrical stimulation of the striatum induced long plateau potentials in reticulospinal cells associated with locomotor episodes. Moreover, activation or inhibi-
tion of the DLR, respectively, facilitated or inhibited the effects of the striatal stimulation, which implies that DLR contributed to the initiation of locomotion. This suggests that the activation of the striatum inhibits the GABAergic projections to the DLR which in turn will lead to a disinhibition of DLR that can then excite the reticulospinal cells which will in turn initiate locomotion. These results indicate thus that DLR is of importance for the effects elicited from striatum, but some of these effects may in addition be exerted via MLR. In addition, the stimulation of the striatum seems to be more effective in the disinhibition the DLR than an injection of the GABA<sub>A</sub> antagonist into the DLR because we were able to get locomotor activity on stimulation. This could be due to the electrical stimulation used at the level of the striatum that could have activated some fibers of passage or nearby structures. However, an injection of α-glutamate into the striatum also succeeds in inducing locomotion (personal observation).

A further understanding of the interaction between striatum and the presumed pallidal structures will, however, require detailed studies of the synaptic action exerted by striatum on the pallidal GABAergic neurons, and recordings of their pattern of activity at rest and during initiation of motor behavior.

Concluding remarks

DLR and MLR can both serve as command regions for locomotion. They both contain neurons that bilaterally project to reticulospinal neurons in the middle and posterior reticulospinal nuclei that will turn on the spinal networks that generate the locomotor movements. DLR and MLR appear both to be subject to a GABAergic tonic inhibition under resting conditions because GABA<sub>A</sub> antagonists will trigger or enhance the occurrence of locomotor activity. Both structures receive long GABAergic projections from a group of cells in the caudoventral part of the medial pallium near eminentia thalami and from another group in the ventrolateral part of the lateral pallium. These GABAergic structures may correspond to the basal ganglia output nuclei in other vertebrates, and the motor effects elicited by stimulation of the striatum may be channeled via these nuclei.

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