Locomotor-related activity of GABAergic interneurons localized in the ventrolateral region in the isolated spinal cord of neonatal mice

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Nishimaru H, Sakagami H, Kakizaki M, Yanagawa Y. Locomotor-related activity of GABAergic interneurons localized in the ventrolateral region in the isolated spinal cord of neonatal mice. J Neurophysiol 106: 1782–1792, 2011. First published July 6, 2011; doi:10.1152/jn.00385.2011.—Inhibitory neurons are an essential element of the locomotor network in the mammalian spinal cord. However, little is known about the firing pattern and synaptic modulation during locomotion in the majority of them. In this study, we performed whole cell recording in visually identified ventrolaterally located GABAergic neurons (VL-GNs) in the rostral (L2 segment) and caudal (L5 segment) lumbar cord using isolated spinal cord preparations taken from glutamate decarboxylase 67-green fluorescent protein (GAD67-GFP) knock-in mouse neonates. These neurons did not respond to electrical stimulation of the ventral root, indicating that they were not Renshaw cells. Ninety-five percent of VL-GNs in the L2 segment and fifty percent of those in the L5 segment showed significant rhythmic firing during locomotor-like rhythmic activity induced by bath application of 5-HT and NMDA. Seventy percent of these neurons fired mainly during the extensor phase, and twenty-five percent fired mainly during the flexor phase. Voltage-clamp recordings revealed that most of these neurons received rhythmic inhibition during the nonfiring phase and excitatory synaptic inputs during the firing phase. Morphological examination of recorded neurons filled with neurobiotin showed that their soma was located lateral to the motoneuron pool and that they extended their processes into the local ipsilateral ventromedial region and dorsal regions. The present study indicates that these GABAergic interneurons located in the ventrolateral region adjacent to the motoneuron pool are rhythmically active during locomotion and involved in the inhibitory modulation of local locomotor network in the lumbar spinal cord.

RHYTHMIC MOTOR OUTPUT underlying mammalian locomotion is generated by the neuronal circuits localized in the spinal cord (Grillner and Wallen 1985). These circuits generate the rhythmic firing of each motoneuron, coordinated activity between flexor- and extensor-related motoneurons, and synergy between the left and right sides of the body. Inhibitory synaptic transmission mediated by glycine and GABA plays crucial roles in the locomotor circuits such as coordinating the timing and the duration of the firing of motoneurons innervating each muscle of each joint in the limbs (for recent review see Kiehn 2006; Nishimaru and Kakizaki 2009). In particular, they are thought to be important for the coordination between antagonistic muscles in the left and right limbs and control of speed during locomotion.

The in vitro isolated spinal cord preparation from neonatal rodents has been used extensively to examine the neuronal mechanism of the locomotor circuits (Cazalets and Bertrand 2000; Kiehn 2006; Schmidt and Jordan 2000; Whelan 2003). This preparation can generate locomotor-like rhythmic motor activity when stimulated by neuroactive substances such as N-methyl-d-aspartate (NMDA) and serotonin [5-hydroxytryptamine (5-HT)]. A previous lesion study utilizing this preparation showed that in a spinal cord of which the dorsal part is removed, an alternating rhythmic activity between the flexor and extensor and between the left and right side can be induced (Kjaerulff and Kiehn 1996), indicating that the essential part of the locomotor circuits is localized in the ventral region. However, the identity of ventral inhibitory interneurons involved in the locomotor activity has not been clear, with a few exceptions. In the ventral spinal cord, Renshaw cells and Ia inhibitory interneurons are two of the best-described inhibitory interneurons (for recent review see Alvarez and Fyffe 2007; Brownstone and Bui 2010). Extracellular recordings in the cat spinal cord showed that both of these ipsilateral projecting neurons that directly inhibit motoneurons are rhythmically active during fictive locomotion (McCrea et al. 1980; Pratt and Jordan 1987). However, how the activity of the rest of the inhibitory neuronal population is synaptically modulated during locomotion in the ventral region is poorly understood. In particular, synaptic modulation of ipsilaterally projecting inhibitory interneurons in the ventral region during locomotor activity has not been examined in detail apart from Renshaw cells (Nishimaru et al. 2006).

In this study, we investigated the firing pattern and synaptic modulation of GABAergic inhibitory interneurons in the ventrolateral region in isolated spinal cord taken from neonatal mice during locomotor-like rhythmic activity. By utilizing the glutamate decarboxylase 67-green fluorescent protein (GAD67-GFP) knock-in mouse to facilitate the identification of GABAergic neurons (Nishimaru et al. 2006, 2010), we recorded the electrical activity of a previously undescribed population of inhibitory interneurons that is located lateral to the motoneuron pool in the rostral and caudal lumbar cord. These neurons were rhythmically active during the locomotor-like activity and were likely to be projecting their axon to the ipsilateral side. The majority of neurons fired mainly at the end...
METHODS

Animal experiments were carried out in a humane manner after approval from the Institutional Animal Experiment Committee of the University of Tsukuba and in accordance with the Institutional Regulation for Animal Experiments and Fundamental Guideline for Proper Conduct of Animal Experiment and Related Activities in Academic Research Institutions under the jurisdiction of the Ministry of Education, Culture, Sports, Science and Technology.

Retrograde labeling of lumbar motoneurons. Heterozygote GAD67-GFP knock-in mouse postnatal day (P) 0–2 neonates (Tamamaki et al. 2003) were anesthetized with isoflurane, eviscerated, and ventrally dissected, and the spinal cord was removed as previously described (Nishimaru et al. 2010). After the spinal cord was placed in the incubation chamber, lumbar motoneurons were retrogradely labeled by applying crystals of rhodamine dextran-amine (Molecular Probes, Eugene, OR) to the cut ventral root (VR) (Nishimaru et al. 2005). The preparations were further processed as previously described (Nishimaru et al. 2006). Motoneuron activity during locomotion was monitored in the L2 and L5 VR on the same side at which the soma of the recorded neuron was located. The major locomotor burst in L2 represents flexor activity while L5 represents extensor in the mouse (Whelan et al. 2000).

Data analysis. Analysis of rhythm firing of ventrolaterally located GABAergic neurons (VL-GNs) during locomotor-like VR activity was performed off-line with Clampfit 10.2 (Molecular Devices) and Spike2 5.1 (Cambridge Electronic Design, Cambridge, UK) software. Circular statistics were performed as previously described (Nishimaru et al. 2006). Briefly, 20 VR bursts taken from a sample of stable 20 locomotor cycles were analyzed. The latency of each spike in the recorded neuron from the onset of the VR burst was measured in relation to the duration of the VR burst (P) to give the phase value (see Kjaerulff and Kiehn 1996 for details). The mean of at least 20 P was calculated with the formula outlined by Kjaerulff and Kiehn (1996). This calculation gives the preferred phase of firing (the mean vector) and the concentration of firing around the preferred phase of firing (expressed by r). A preferred phase of firing from 0 to -0.5 means that the recorded GABAergic neuron fired in phase with the reference VR, while phase values with a direction in the range 0.5 to 1.0 corresponded roughly to cells firing out of phase with the VR. These data are presented in circular plots. P values for the significance of r (P < 0.05) were taken from Zar (1974). The preferred phase of firing shown in the circular plots is the mean vector of all the spikes calculated from single reference analysis. To examine the firing frequency of the recorded neurons during locomotion, the phase of each spike during 20 cycles was normalized by the duration of each locomotor cycle period in the referred VR. The normalized cycle periods were subdivided into 10 bins, the first 5 representing the active phase in the reference VR and the last 5 equivalent to the nonactive phase (see Fig. 2, D and G). The average firing frequencies in each bin were used to create a spike frequency histogram.

The synaptic currents were examined by voltage-clamp recording. The membrane potential was held at -40 mV and -60 mV to examine the inhibitory postsynaptic currents (IPSCs) and excitatory postsynaptic currents (EPSCs), respectively (Nishimaru et al. 2006, 2010). Detection and analysis of synaptic events were performed off-line with Clampfit 10.2. The postsynaptic currents (PSCs) during locomotor-like activity recorded in voltage clamp were first semiautomatically detected by template matching-based detection before the events in the whole recorded trace were visually checked. The frequency and the amplitude of the PSCs during the locomotor phase were calculated in each phase bin of the normalized locomotor cycle. To compare between different neurons, values of each bin were normalized and averaged with those of the bin before and after (e.g., the value of bin / is an average of normalized values of bins 10, 1, and 2; see Figs. 6 and 8). Statistical comparisons were performed by Student’s t-tests, and summary statistics report means ± 1 SD unless otherwise specified.

Visualization of neurobiotin-labeled neurons. We labeled the neurons with 0.2–0.3% neurobiotin for a 30- to 90-min duration. All the preparations containing a labeled cell were fixed in 4% paraformaldehyde in 0.1 M PBS overnight at 4°C. Thirty neurons located in the L2 segment were successfully recovered for further analysis. We

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examined the expression of calbindin in 5 neurons and morphology in
the remaining 25 neurons. For calbindin immunohistochemistry, the
preparations were cryoprotected with 30% sucrose in 0.1 M PB and
cut into 50-μm-thick transverse sections. The sections were incubated
overnight with Alexa-conjugated streptavidin (Invitrogen, Carlsbad
CA) to visualize the labeled cell with the anti-calbindin antibody as
described above.

For examination of the morphology of the recorded neuron, the
fixed preparations were incubated in 1% avidin-biotin peroxidase
complex solution (Vectastain ABC Elite kit; Vector Laboratories,
Burlingame, CA) in PBS containing 0.1% Triton X-100 overnight
at room temperature and then in 0.01% diaminobenzidine tetrahy-
drochloride for 20 min. After cryoprotection in 0.1 M PB with 30%
sucrose, the preparations were cut into 50-μm-thick transverse
sections and mounted on slides with 2% gelatin. Neurons were
visualized on a light microscope, traced by camera lucida, scanned
in, and retraced in Illustrator CS (Adobe Systems).

RESULTS

In the ventral horn of the mouse neonatal spinal cord, the
soma of the GABAergic neurons could be observed surrounding
the motoneuron pool (Fig. 1, A–C). In previous studies, the
majority of Renshaw cells, immunoreactive to the calcium-
binding protein calbindin D28k, were shown to be clustered in
the ventromedial part of the ventral horn and relatively sparse
in the ventrolateral region (Alvarez et al. 2005; Mentis et al.
2006; Siembab et al. 2010). We also observed calbindin-
positive cells in the ventromedial area close to the surface in
the GAD67-GFP spinal cord (Fig. 1, D–H). We noted that

![Fig. 1. Identification of ventrolaterally lo-
cated GABAergic neurons (VL-GNs) in the
glutamate decarboxylase 67-green fluorescent
green protein (GAD67-GFP) mouse spinal
cord. A–F: transverse sections of lumbar spi-
nal cord showing the distribution of GAD67-
GFP-positive cells in the ventral region. Note
that motoneurons (MNs) labeled by rhoda-
mine dextran (B, magenta) were surrounded
by GFP-positive GABAergic neurons (A,
green). Double immunostaining of the spinal
cords with anti-GFP (D, green) and anti-
calbindin D28K (E, magenta) shows the lo-
cations of the VL-GNs (arrowheads) and a
group of Renshaw cells (RCs) (yellow ar-
rows) in the ventromedial region. A merged
image (F) includes nuclear counterstaining
with DAPI (blue). G: a magnified image of
calbindin-positive cells indicated by a single
asterisk in F. H: a magnified image of VL-
GNs indicated by double asterisks in F.
I: responses to ventral root (VR) stimulation
(VR stim) in VL-GNs. The majority of VL-
GNs did not respond to VR stim (top), while
a few neurons received inhibitory inputs (n =
2, bottom). The mean latency of the response
was 6.7 ms. All traces are of 5 superimposed
K: unlike Renshaw cells (yellow arrows),
VL-GNs (arrowheads) were not labeled by
anti-calbindin staining. Scale bars = 50 μm
in A–F and K; 20 μm in H.
GFP-positive GABAergic neurons were also distributed in the lateral part of the motoneuron pool (Fig. 1, D–F). Double immunostaining revealed that only 1.8% (5 of 267 cells from 4 spinal cords) were immunoreactive for calbindin D28k, indicating that the majority of these GABAergic neurons are not Renshaw cells. In this study we focused on these non-Renshaw cell GABAergic populations located laterally to the motoneuron pool.

Utilizing visually guided patch-clamp recording in the isolated spinal cord preparation, we looked for GFP-positive neurons located lateral to the motoneuron pool in the isolated spinal cord preparation. After establishing whole cell record-
ing, we electrically stimulated the VR of the same segment to the soma with the intensity that evoked bursting excitatory response in Renshaw cells and recurrent inhibition in motoneurons in previous studies (Fig. 1; Nishimaru et al. 2006, 2010). Only 5 of 77 patched cells responded to the stimuli with an excitatory response with latencies (2–3 ms) similar to Renshaw cells recorded in the previous study with a similar preparation (data not shown; Nishimaru et al. 2010). In two cells, the VR stimulation evoked an inhibitory response with latencies (6–8 ms) similar to the recurrent inhibition observed in motoneurons (Nishimaru et al. 2010; Fig. 1). We did not include these cells in the present study and examined the electrical activity of those neurons that did not respond to the stimulus (n = 70). Furthermore, we confirmed that neurons labeled with neurobiotin were not immunoreactive for calbindin (Fig. 1, J and K; n = 5). These results indicate that VL-GNs form a population distinct from the Renshaw cells.

Firing patterns of VL-GNs during chemically induced locomotion. We examined the electrical activity of VL-GNs during the locomotor-like rhythmic activity induced by bath application of NMDA and 5-HT (Fig. 2A). The rhythmic activity was monitored in the VR of the L2 segment (L2VR) and that of the L5 segment (L5VR), ipsilateral to the side of the recorded neuron. The main bursts of L2VR during locomotor-like rhythmic activity in rodents represent flexor motoneuronal activity, while those of L5VR represent activity of the extensor-related motoneurons (Kiehn and Kjaerulff 1996; Whelan et al. 2000). In this preparation, previous studies have shown that the rhythmogenic capability is higher in the rostral part than the caudal part of the lumbar spinal cord (Cazalets et al. 1995; Kjaerulff and Kiehn 1996). We therefore first recorded neurons located in the L2 segment (n = 40). When the locomotor-like rhythmic activity was induced in the VRs, rhythmic oscillation accompanied by action potentials was observed in VL-GNs (Fig. 2A). Typically, L2 VL-GNs fired few spikes (1–10, mean 2.6 ± 1.4 spikes) during a single locomotor cycle. Examples of the firing pattern are shown in Fig. 2 for two L2 VL-GNs that fired mainly during the extensor phase (Fig. 2, B–D) and the flexor phase (Fig. 2, E–G), respectively. The quantitative measurement of the preferred locomotor phase of firing was first examined by circular statistics (Fig. 2, C and F). We used Rayleigh’s test (Zar 1974) to determine whether the timing of the spike was clustered significantly (P < 0.05) in this plot. More than 90% of the VL-GNs located in L2 showed a significant preferred spiking phase (37 of 40 cells). Seventy percent of the recorded L2 VL-GNs (28 of 40 cells) fired out of phase with the L2 burst, while 22.5% (9 of 40 cells) fired in phase with the L2 burst, indicating that the majority of them fire during the extensor phase. This was confirmed by spike frequency histograms in which each locomotor cycle is normalized and divided into 10 bins (Fig. 2, D and G). Like the circular statistics, we found with this analysis that VL-GNs fired throughout the preferred firing phase. Interestingly, the highest firing frequency was observed at the end of the flexor phase or at the beginning of the extensor phase.

We next examined the electrical activity of caudally located VL-GNs in the L5 segment (n = 30) to see whether they are rhythmically active as well. The circular statistics showed that half of the L5 neurons recorded did not have a significant preferred phase of firing (15 of 30 cells). Of the 15 cells that showed a particular preferred firing phase, 11 cells fired in phase with the L5 activity (Fig. 3, A–C) while 4 cells were active out of phase during locomotor-like rhythmic activity (Fig. 3, D–F). VL-GNs in L5 fired 1–15 spikes (mean 5.4 ± 2.8 spikes) during a single locomotor cycle. The results of the circular statistics of rostral and caudal VL-GNs are summarized in Fig. 4. In the L2 segment, most neurons had the preferred phase of firing around 0.25–0.75 in the normalized locomotor phase, which corresponds to the late part of the flexor phase to the early part of the extensor phase. The majority of VL-GNs located in the L5 segment had the preferred phase of firing scattered throughout the extensor phase. Interestingly, the rhythmically active VL-GNs in L5 showed significantly lower r values (mean 0.46 ± 0.12) compared with those of L2 VL-GNs (mean 0.63 ± 0.02, P < 0.01; Fig. 4C). These results indicate that the majority of the VL-GNs in the

![Fig. 4. Summary of the preferred firing phase of VL-GNs. A: circular plot showing the mean firing phase of L2 VL-GNs. B: circular plot showing the mean firing phase of L5 VL-GNs. The dashed line in A and B indicates significance (P < 0.05). C: histogram of the mean r value of the VL-GNs in L2 and L5 that showed significance. *P < 0.05.](http://jn.physiology.org/10.1152/jn.00665.2011)
lumbar spinal cord are rhythmically active (53 of 70 cells) and most of them fired during the late flexor phase and early extensor phase. However, the activity of VL-GNs localized in the rostral segment was modulated strongly compared with those in the caudal segment during locomotor-like activity.

**Synaptic inputs modulating the VL-GN.** Rhythmically active spinal interneurons receive inhibitory and/or excitatory synaptic inputs (Dougherty and Kiehn 2010; Nishimaru et al. 2006; Wu et al. 2011; Zhong et al. 2010). We next examined the synaptic inputs underlying the rhythmic firing of L2 VL-GNs during the locomotor cycle in voltage-clamp mode. When the membrane potential was held at $-40 \text{ mV}$, inhibitory inputs appeared as outward currents since the reversal potential of $\text{Cl}^-$-mediated IPSCs is around $-78 \text{ mV}$ under our experimental conditions (Nishimaru et al. 2010). In extensor-related VL-GNs that fired at the end of the flexor burst to the extensor phase ($n = 21$; Fig. 5, A and B), rhythmic IPSCs were observed (Fig. 5, C and D). In these neurons a barrage of outward currents was observed mainly during the nonfiring phase. The maximum frequency of IPSCs in a phase bin was $17.2 \pm 6.3$ Hz (range 4.7-26.6 Hz), while the maximum amplitude was $64.0 \pm 32.2 \text{ pA}$ (range 28.5-145.9 pA). The minimum IPSC frequency during the locomotor cycle was typically observed during the firing phase of the neuron [frequency $6.0 \pm 5.0$ Hz (range 1.1-19.4 Hz)], while the minimum amplitude was $30.0 \pm 18.3 \text{ pA}$ (range 10.5-85.1 pA). When the membrane potential was held at $-60 \text{ mV}$, EPSCs that appeared as inward currents were most prominent during the extensor phase (Fig. 5, E and F). The maximum EPSC frequency was $16.5 \pm 9.0$ Hz (range 4.6-44.3 Hz), and maximum amplitude was $-31.2 \pm 16.2 \text{ pA}$ (range $-13.3$ to $-77.3 \text{ pA}$). The minimum EPSC frequency was $7.4 \pm 6.7$ Hz (range 0.0-26.0 Hz), while the minimum amplitude was $-17.5 \pm 8.7 \text{ pA}$ (range 0.0 to $-37.1 \text{ pA}$). The normalized frequency and amplitude of both of the PSCs during the locomotor cycle in 21 extensor-related VL-GNs are summarized in Fig. 6. In all cells we observed prominent inhibitory synaptic inputs during the nonactive phase (Fig. 6A). IPSCs in these neurons showed such phasic changes in frequency ($n = 1$), amplitude ($n = 3$), or both ($n = 17$). The majority of the extensor-related VL-GNs (19 of 21...
cells) mainly received excitatory synaptic inputs during the active phase during the locomotor cycle. EPSCs in these neurons showed such phasic changes in frequency \((n = 1)\), amplitude \((n = 9)\), or both \((n = 9)\).

In flexor-related VL-GNs \((n = 7)\) that fired predominantly in phase with the L2 VR burst (Fig. 7, A and B), IPSCs were less pronounced during the firing phase (Fig. 7, C and D). The maximum IPSC frequency was 18.9 ± 7.4 Hz (range 13.0–32.8 Hz), and the maximum amplitude was 82.7 ± 52.5 pA (range 24.1–190.5 pA) in these neurons. The minimum IPSC frequency during the locomotor cycle was 9.4 ± 8.1 Hz (range 1.7–26.5 Hz), and the minimum amplitude was 54.7 ± 39.7 pA (range 12.5–133.9 pA). In these neurons, the EPSCs was most prominent during the flexor phase. The maximum EPSC frequency of these neurons was 28.4 ± 10.2 Hz (range 15.4–47.0 Hz), and maximum amplitude was −52.5 ± 35.4 pA (range −21.3 to −126.3 pA). The minimum EPSC frequency of these neurons was 15.5 ± 6.6 Hz (range 7.3–23.7 Hz), and minimum amplitude was −35.6 ± 21.4 pA (range −14.0 to −78.6 pA). As summarized in Fig. 8, most flexor-related VL-GNs received inhibitory synaptic inputs mainly during the nonfiring phase and excitatory inputs during the firing phase. IPSCs in these neurons showed such phasic changes in frequency \((n = 1)\), amplitude \((n = 3)\), or both \((n = 9)\) (Fig. 8A). Six of seven flexor-related VL-GNs received phasic EPSCs during the active phase (Fig. 8B). EPSCs in these neurons showed such phasic changes in amplitude \((n = 1)\) or both frequency and amplitude \((n = 5)\).

**Morphology of VL-GNs.** Finally, we examined the anatomic characteristics of recorded VL-GNs. The neurons were filled with neurobiotin for 30–90 min during the recordings and stained with diaminobenzidine after fixation. We successfully filled and recovered 25 VL-GNs to examine morphology (Fig. 9). The soma of all the labeled cells were located in the lateral ventral horn. Axons were never seen crossing the midline, suggesting that VL-GNs may predominantly be ipsilaterally projecting neurons. Their processes spread along the dorsoventral axis in a columnar orientation.

In most of the neurons \((n = 22)\) the processes were extended to the ventromedial region, where many neurons related to the locomotor CPG are located (Fig. 9C; Kjaerulff and Kiehn 1996). Of all neurons examined, 21 neurons had their processes extending into the intermediate zone. Such a feature could be observed in extensor- or flexor-related VL-GNs as well as neurons that did not have a clear preferred firing phase, and we could not find clear morphological distinction between these neurons.

**DISCUSSION**

This study describes the electrophysiological and morphological analyses of GABAergic interneurons located in the ventrolateral region in the lumbar spinal cord, with focus on the firing pattern and synaptic modulation during locomotor-like rhythmic motor activity. The majority of the VL-GNs recorded were rhythmically active, particularly at the extensor phase during locomotion. These neurons received reciprocal excitatory and inhibitory synaptic inputs that are likely to be determining the timing of firing. The activity of VL-GNs localized in the rostral segment was modulated more strongly than that of those in the caudal segment.

**VL-GNs are an inhibitory neuronal population distinct from Renshaw cells.** In the mammalian ventral spinal cord, Renshaw cells and Ia inhibitory interneurons are two of the few functionally identified GABAergic interneurons that project their axons ipsilaterally (for recent review see Brownstone and Bui 2001). Renshaw cells expressing calbindin have been shown to be mainly located in the ventromedial region in the neonatal spinal cord (Mentis et al. 2006). In this study we recorded from GABAergic neurons that are located in the ventrolateral region, where we detected very few calbindin-positive cells. In this preparation, we were able to electrophysiologically distinguish VL-GNs from Renshaw cells since they did not have detectable excitatory input from adjacent motoneurons (Mentis et al. 2006; Nishimaru et al. 2006). The recorded neurons were...
not calbindin positive, although we cannot completely rule out the possibility that the calcium-binding protein was dialyzed out during the prolonged whole cell patch-clamp recording. In the cat spinal cord, Ia inhibitory interneurons that mediate reciprocal inhibition between the motoneurons innervating antagonistic muscles in the reflex pathway (Eccles et al. 1956; Jankowska and Roberts 1972) have been shown to be rhythmically active during fictive locomotion (Pratt and Jordan 1987). The electrophysiological and morphological nature of Ia inhibitory interneuron has not been examined in detail in the rodent spinal cord at present, and we cannot completely rule out the possibility that VL-GNs include these interneurons. However, many of the Ia inhibitory interneurons are located in lamina VII, dorsal or dorsomedial to the motoneuron pool in cat (Jankowska and Lindstrom 1972) and in mouse (Alvarez et al. 2005), while the GABAergic neurons recorded in this study are located in the ventrolateral region. Moreover, since it has been shown that Ia inhibitory interneurons receive direct inhibition from Renshaw cells (Hultborn et al. 1971; Wang et al. 2008), we have discarded neurons that were inhibited by VR stimulation in this study.

Rhythmic firing pattern of VL-GNs during locomotion. Most of the VL-GNs were rhythmically active during locomotor-like activity, with more than three-fourths of them showing a significant preferred firing phase. In the L2 segment >70% of the VL-GNs fired predominantly during the extensor phase. This is in contrast to the Renshaw cells located in the same segment, 50% of which fired in phase with the flexor-related rhythm while the rest fired in the extensor phase (Nishimaru et al. 2006). The extensor-related VL-GNs located in the L2 segment mainly fired in the middle of the locomotor cycle, which timing corresponds to the end of the flexor burst to the beginning of the extensor burst. Interestingly, most of the flexor-related VL-GNs in the L2 also had a preferred phase of firing in the latter half of the flexor phase, indicating that these neurons exert their inhibitory effects during the transition between the flexor phase and the extensor phase. Since the VR activity in L2 represents the flexor activity, some of these
neurons might be involved in the burst termination of the dominant motoneuronal activity in this segment. Half of the L5 VL-GNs did not have a significant preferred phase of firing, and even the neurons that were firing rhythmically tended to have smaller r values compared with neurons localized in the L2 segment. Moreover, the L5 VL-GNs had more scattered preferred phase values, indicating that they are more loosely tuned than the L2 VL-GNs.

This may be due to the weaker rhythmogenec capability of the caudal lumbar segment compared with the rostral segment in this isolated spinal cord preparation as previously described (Cazalets et al. 1995; Kjaerulff and Kiehn 1996). Similar observations were reported in previous studies of interneurons located in the ventromedial region (Tresch and Kiehn 1999).

The electrical activity of VL-GNs was modulated by phasic inhibitory synaptic inputs whose amplitude and frequency were high at the beginning of the flexor phase and at the end of the extensor phase. These inputs are likely to be preventing VL-GNs from firing at these times. Many of the VL-GNs appeared to receive the strongest excitatory synaptic inputs during the firing phase, determining the timing of the rhythmic firing in these cells. Such alternating excitatory and inhibitory synaptic inputs during a locomotor cycle have been observed in motoneurons (Cazalets et al. 1996; Endo and Kiehn 2008; Hochman and Schmidt 1998) and interneurons (Raastad et al. 1996) in similar preparations as well as in vivo cat preparations (Orsal et al. 1986; Shewchuk and Jordan 1985). In a previous study, it was shown that Renshaw cells receive rhythm synaptic inhibition at the firing phase during locomotion. By contrast, in most of the VL-GNs recorded in this study the frequency and the amplitude of the rhythmic IPSC peaked during the nonfiring phase, indicating that the activity of these two types of GABAergic interneurons in the ventral horn is synaptically modulated by the locomotor network in a distinctive manner.

GABAergic neurons in the spinal locomotor network. It has been shown in previous studies that GABAergic inhibitory synaptic transmission is involved in the generation of the locomotor pattern in the mammalian spinal cord. Blocking fast synaptic transmission mediated by GABA_A receptors prolonged the burst duration of motoneurons during locomotor-like activity (Hinckley et al. 2005; Pflieger et al. 2002). Recent studies that took advantage of the genetically driven labeling of neurons by fluorescent proteins have examined GFP-positive GABAergic interneurons localized in the dorsal horn (Wilson et al. 2010) and commissural GABAergic neurons located in lamina VIII (Wu et al. 2011) as well as Renshaw cells (Nishimaru et al. 2006). These studies showed that these inhibitory interneurons fired rhythmically with a preferred phase of firing and possibly playing a role in control of timing and duration of the rhythmic motor outputs during locomotion. However, the actual functional role of most of these GABAergic interneurons remains unclear. One inhibitory internuron class whose role has been investigated is the V1 interneurons. These interneurons derive from progenitor cells transiently expressing the transcription factors paired box gene 6 (Pax6) and engrailed 1 (En1) during their postmitotic stage (Ericson et al. 1997; Sapir et al. 2004). This interneuron class gives rise to ipsilaterally projecting inhibitory interneurons including Renshaw cells and Ia inhibitory interneurons in the postnatal animal (Alvarez et al. 2005). These neurons also appear to give rise to some of the interneurons located in the ventrolateral region as well (Gosgnach et al. 2006). When the firing activity of these neurons was blocked, the cycle period of the locomotor-like rhythm was reversibly prolonged while the coordination between the flexor and extensor motoneurons persisted (Gosgnach et al. 2006). This finding indicates that V1 interneurons are involved in controlling the duration of the motor burst and that there are other neuronal populations involved in the coordination of flexor and extensor activity. In this study, we were not able to determine whether the VL-GNs belong to this interneuronal population since the transcription factors that serve as molecular markers cannot be detected in postnatal animals by immunohistochemistry and further studies utilizing transgenic mice expressing reporter proteins are required.
Conclusions. In this study, we showed that VL-GNs are inhibitory neurons that do not belong to the Renshaw cell population. These neurons were rhythmically active during locomotion, and the majority of them fired in the middle of the locomotor cycle. Their activity is modulated by reciprocal excitatory and inhibitory synaptic inputs. Morphological examination of these neurons indicated that most of them are likely to be ipsilaterally projecting neurons. Some of these locomotor-related GABAergic interneurons that are located adjacent to the motoneuron pool could be providing inhibition during the transition of the flexor-extensor phase.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

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