Asymmetric Operation of the Locomotor Central Pattern Generator in the Neonatal Mouse Spinal Cord

Toshiaki Endo and Ole Kiehn

Mammalian Locomotor Laboratory, Department of Neuroscience, Karolinska Institutet, Stockholm, Sweden

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Endo T, Kiehn O. Asymmetric operation of the locomotor central pattern generator in the neonatal mouse spinal cord. J Neurophysiol 100: 3043–3054, 2008. First published October 1, 2008; doi:10.1152/jn.90729.2008. The rhythmic voltage oscillations in motor neurons (MNs) during locomotor movements reflect the operation of the pre-MN central pattern generator (CPG) network. Recordings from MNs can thus be used as a method to deduce the organization of CPGs. Here, we use continuous conductance measurements and decomposition methods to quantitatively assess the weighting and phase tuning of synaptic inputs to different flexor and extensor MNs during locomotor-like activity in the isolated neonatal mice lumbar spinal cord preparation. Whole cell recordings were obtained from 22 flexor and 18 extensor MNs in rostral and caudal lumbar segments. In all flexor and the large majority of extensor MNs the extracted excitatory and inhibitory synaptic conductances alternate but with a predominance of inhibitory conductances, most pronounced in extensors. These conductance changes are consistent with a “push–pull” operation of locomotor CPG. The extracted excitatory and inhibitory synaptic conductances varied between 2 and 56% of the mean total conductance. Analysis of the phase tuning of the extracted synaptic conductances in flexor and extensor MNs in the rostral lumbar cord showed that the flexor-phase–related synaptic conductance changes have sharper locomotor-phase tuning than the extensor-phase–related conductances, suggesting a modular organization of premotor CPG networks consisting of reciprocally coupled, but differently composed, flexor and extensor CPG networks. There was a clear difference between phase tuning in rostral and caudal MNs, suggesting a distinct operation of CPG networks in different lumbar segments. The highly asymmetric features were preserved throughout all ranges of locomotor frequencies investigated and with different combinations of locomotor-inducing drugs. The asymmetric nature of CPG operation and phase tuning of the conductance profiles provide important clues to the organization of the rodent locomotor CPG and are compatible with a multilayered and distributed structure of the network.

INTRODUCTION

Locomotion in limbed animals is a complex motor task involving alternating activities in flexor and extensor muscles within the limb and between muscles on either side of the body. These coordinated muscle activities are for the major part generated by local neuronal networks in the spinal cord, called central pattern generators (CPGs), that synthetically drive motor neurons (MNs) into rhythmic firing. The time-varying synaptic activity impinging onto MNs during locomotor activity reflects the operation of the CPG network and recordings from MNs have been used as a method to deduce the overall organization of locomotor CPGs. Earlier studies of limb locomotor CPGs have shown that MNs receive rhythmic excitatory synaptic inputs in their active phase and inhibitory inputs in their inactive phase during scratching (Perreault 2002; Robertson and Stein 1988) and walking (Burke et al. 2001; Cazale et al. 1996; Hochman and Schmidt 1998; Jordan 1983; Orsal et al. 1986; Schmidt 1994; Schmidt et al. 1988; Shefchyk and Jordan 1985a,b), similar to what has been described for nonlimbed swimming CPGs (Kahn 1982; Russell and Wallen 1983; Wallen et al. 1993). This push–pull organization of synaptic inputs to MNs during rhythmic activity has led to the notion that spinal premotor CPG networks are organized in reciprocally coupled flexor and extensor modules/units that, when active in their appropriate phase, provide excitation to agonist MNs and inhibition to antagonist MNs in a symmetric manner (see Grillner 1981; Jordan 1991; Kiehn 2006; McCrea and Rybak 2008). However, some studies have demonstrated a predominance of inhibitory drive to certain groups of MNs during locomotion (Hochman and Schmidt 1998; Schmidt et al. 1988; Shefchyk and Jordan 1985a) and a possible difference in the relative importance of excitation and inhibition in flexor and extensor MNs (Godderez et al. 1990; see their Figs. 9 and 10), suggesting a more asymmetrical CPG output. Quantitative information of the relative contribution of excitatory and inhibitory synaptic inputs in different MN pools, on the other hand, is lacking and needed to evaluate the possible scale and extent of an asymmetrical output and the consequences for the CPG organization. Moreover, a recent study in in vitro turtle spinal cord preparations reported that excitatory and inhibitory conductances in hindlimb MNs during rhythmic scratch-like network activity varied in-phase with the peak at the active phase of the scratch episodes (Berg et al. 2007). Detection of the balanced excitation and inhibition was conditional on quantifying the phase-related inhibitory and excitatory synaptic conductances and has not been described before in any other locomotor studies. This mode of operation is incompatible with the general concept of a reciprocal organization of flexor-related and extensor-related CPG networks.

In the present study we provide a quantitative assessment of the weighting and phase tuning of synaptic inputs to different flexor and extensor MNs during locomotor-like activity in the isolated neonatal mice lumbar spinal cord preparation. This preparation has become a model system for mammalian locomotion (Bonnot et al. 2002; Cazalets and Bertrand 2000; Kiehn 2006; Kiehn et al. 2000; Nishimaru et al. 2000; Schmidt and Jordan 2000; Whelan 2003). We applied the same conductance measurement and decomposition methods (Anderson et al. 2000; Borg-Graham et al. 1998; Marino et al. 2005; Shu...
et al. 2003) as used in the turtle preparation (Berg et al. 2007) during drug-induced locomotor-like activity. Our results reveal a highly asymmetrical operation of reciprocally organized flexor and extensor CPG modules, with dominance of inhibitory conductances. The asymmetrical operation remained under different modes of operation of the locomotor network. Our data also showed a difference in the operation of distinct CPG modules in the rostral and caudal lumbar segments and provided evidence for a layered architecture of the rodent locomotor CPG.

Parts of this study were previously presented in abstract form (Endo and Kiehn 2007).

METHODS

Electrophysiological recordings

All procedures followed Swedish federal guidelines for animal care and were approved by the local Animal Care and Use Committee. Newborn C57BL6 mice (postnatal days 0–4) were anesthetized with isoﬂurane and the spinal cords were removed as described previously (Butt et al. 2005; Kiehn and Kjaerulff 1996). The preparations were placed in a recording chamber mounted in an upright microscope and continuously perfused with Ringer solution containing (in mM): 111.14 NaCl, 3.09 KCl, 1.1 KH2PO4, 1.26 MgSO4, 2.52 CaCl2, 25 NaHCO3, and 11.1 glucose (pH 7.4, bubbled with 95% O2-5% CO2). Locomotor-like activities were induced by bath application of N-methyl-D-aspartate (NMDA, 5–10 μM), in combination with serotonin (5-HT, 5–10 μM), and recorded with glass suction electrodes as ventral root (VR) activity in lumbar segments (L) 2, 3, or 5. Dopamine (25–50 μM) was also applied in addition to NMDA and 5-HT in some experiments. The main bursts in the L2 and L3 VRs reﬂect ﬂexor activity, whereas the L5 VR activity reﬂects extensor activity (Whelan et al. 2000). The recorded signals were ampliﬁed ×20,000 and bandwidth-ﬁltered at 100–1,000 Hz using a custom-made ampliﬁer. Whole cell patch-clamp recordings were obtained from visually patched motor neurons (Nishimaru et al. 2005) located in the same segments as the recorded VRs. Patch pipettes were ﬁlled with the internal solution, containing (in mM): 138 K-gluconate, 10 HEPES, 5 ATP-Mg, 0.3 GTP-Li, 0.0001 CaCl2, adjusted to pH 7.3 with KOH. Cesium chloride (5 mM) and N-(2,6-dimethylphenyl carbamoylmethyl)-triethylammonium bromide (QX-314, 5–10 mM) were added to the solution to reduce intrinsic K+ and Na+ conductances, respectively. MNs were identiﬁed by antidromic activation from the ventral roots before QX-314 diffused enough to block action potentials. The resistance of the pipettes was in the range of 3–7 MΩ. The access resistance was in the range of 9–20 MΩ during recordings and continuously corrected by balancing the bridge in current-clamp recordings. In voltage-clamp recordings, the series resistance was compensated by about 70%. The signals were recorded with a Multiclamp 700A patch-clamp ampliﬁer (Molecular Devices, Palo Alto, CA). All recordings were performed at room temperature (20–23°C). Only recordings where the MN membrane potential, the access resistance, and the locomotor-like activity remained stable throughout the recording period were included in the analysis.

Data analysis

The methods for conductance measurement and decomposition were basically the same as recently used in turtle scratching preparations by Berg et al. (2007) (Anderson et al. 2000; Borg-Graham et al. 1998; Marino et al. 2005; Shu et al. 2003). To perform the conductance measurements, MNs were recorded during locomotor-like activity (Fig. 1A). Individual locomotor cycles were divided into ﬂexor phase and extensor phase by using the half-amplitude in rectiﬁed and smoothed VR signals as onset and offset reference points. Both phases were then subdivided into ﬁve bins of equal length (Fig. 1B). Phases 1–5 therefore correspond to the ﬂexor phase (active phase of L2 and L3; inactive phase of L5) and phases 6–10 correspond to the extensor phase (inactive phase of L2 and L3; active phase of L5). Only locomotor periods where the cycle period varied <10% throughout the measurements were used for analysis. The mean membrane potential in each bin for the recorded MN was then obtained by averaging values from 5 to 10 locomotor cycles. This procedure was performed for recordings obtained at two or three diﬀerent injection current levels and the total conductance was calculated for each bin as the slope of the current–voltage relationship obtained by linear regression (Fig. 1C). The decomposition of the conductances was based on the following equations:

$$G_{total} = G_e + G_i + G_{leak}$$

$$I_{inj} = G_e(V_m - E_e) + G_i(V_m - E_i) + G_{leak}(V_m - E_{leak})$$

where $G_{total}$ is the total conductance; $G_e$ is the excitatory conductance; $G_i$ is the inhibitory conductance; $G_{leak}$ is the leak conductance; $I_{inj}$ is the injected current; $V_m$ is the membrane potential; and $E_e$, $E_i$, and $E_{leak}$ are the reversal potentials for excitatory, inhibitory, and leak currents, respectively. To extract $G_e$ and $G_i$ from these simultaneous equations, we determined the remaining parameters as follows: $G_{total}$, $I_{inj}$, and $V_m$ were determined as described earlier for each bin and $E_i$ was determined as mean values for the measured reversal potentials of spontaneous inhibitory postsynaptic potentials (IPSPs), −74.8 mV (corrected for liquid junction potential) ($n = 6$). The reversal potential for $E_e$ is slightly more hyperpolarized than commonly reported for IPSPs recorded in rodent MNs (Cazalet et al. 1996; Hochman and Schmidt 1998) due to the electrode solution used in the present experiments. $E_e$ was set to the commonly accepted value of 0 mV. Assuming that $G_{leak}$ and $E_{leak}$ were time- and voltage independent, these parameters were set to arbitrary constant values (see following text). Although we could easily determine the actual values for $G_{total}$ and $E_{leak}$ in the nonlocomoting preparations, we have no way to determine these values during locomotor-like activity. Consequently, we could not determine the absolute values of $G_e$ and $G_i$. We instead calculated the relative changes of $G_e$ ($ΔG_e$) and $G_i$ ($ΔG_i$) as a percentage of the mean value of $G_{total}$ (mean $G_{total}$) averaged over the locomotor cycle. In this calculation, the shape and amplitude of $ΔG_e$ and $ΔG_i$ proﬁles are insensitive to the precise value of $E_{leak}$ and $E_{leak}$. The membrane potential was corrected by a liquid junction potential of −10 mV.

To analyze the preferred phase of the conductances, the normalized modulation amplitude of the extracted conductance ($ΔG$ divided by maximum amplitude of $ΔG$, $ΔG/\max ΔG$) was plotted against the locomotor phase in polar coordinates for individual motor neurons. To evaluate mean phase and sharpness of the phase tuning of the conductance proﬁles, all vectors were summed and normalized by the sum of the length of all vectors. The direction of the resultant vector indicates the mean active phase angle of the conductance. The length of the resultant vector represents the sharpness of the phase tuning. The resultant length of a conductance that increases only in one phase will be 1, whereas that of a conductance that increases equally in symmetrically distributed bins will be 0.

Summary statistic values are given as means ± SE. Paired and unpaired t-tests, one-way ANOVA, and Games–Howell test were used to assess statistical signiﬁcance. Differences were considered to be signiﬁcant at $P < 0.05$.

Drugs

NMDA, 5-HT, 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX), strychnine hydrochloride, picrotoxin, and dopamine were purchased from Sigma (St. Louis, MO). Tetrodotoxin (TTX) was purchased from Tocris Cookson (Bristol, UK). QX-314 was purchased from RBI (Natick, MA).
RESULTS

Motor neuron database

Whole cell patch-clamp recordings were obtained from a total of 40 lumbar motor neurons (MNs). Locomotor-like activity was induced by bath application of a combination of NMDA and 5-HT (5–10 μM) with \( n = 6 \) MNs or without \( n = 34 \) dopamine (25–50 μM), and monitored by recording the ventral root activity in lumbar ventral root L2/L3, representing flexor activity, or L5, representing extensor activity (Whelan et al. 2000). MNs were classified as flexor or extensor MNs on the phase during which the membrane potential \( V_m \) was most depolarized. Twenty-two MNs were classified as flexors and 18 as extensors. All flexor MNs (\( n = 19 \) without dopamine; \( n = 3 \) with dopamine) and 6 extensor MNs (all without dopamine) were recorded in the L2/L3 segments, whereas 12 extensor MNs (\( n = 9 \) without dopamine; \( n = 3 \) with dopamine) were recorded in the L5 segment. None of the recorded MNs in L5 showed out-of-phase oscillations with the L5 root bursts.

The mean locomotor cycle length in all experiments was 2.4 ± 0.1 s, with no significant difference between the data groups (one-way ANOVA, \( P = 0.17 \)).

Measurements of conductance changes

To derive the total conductance \( G_{\text{total}} \) profile over the locomotor cycle the average \( V_m \) was calculated for recordings at two or three different injection current \( I_{\text{inj}} \) levels in normalized cycles where bins 1–5 define the flexor phase and bins 6–10 the extensor phase (Fig. 1, A and B). The \( G_{\text{total}} \)-phase trace was derived from the slope of the linear regression line for each phase bin. We calculated and derived the variation of total, excitatory, and inhibitory conductances (\( \Delta G_{\text{total}}, \Delta G_e, \text{ and } \Delta G_i \), respectively) from the \( G_{\text{total}} \)-trace as a percentage of the mean value of the \( G_{\text{total}} \) averaged over the locomotor cycle. Note that \( \Delta G_{\text{total}}, \Delta G_e, \text{ and } \Delta G_i \) show phase-dependent changes only, not including tonic components of the conductances. The excitation increased during flexor phase, whereas the inhibition varied out of phase with the excitation.

FIG. 1. Alternating excitatory and inhibitory conductance in flexor motor neuron (MN) during serotonin (5-HT)/N-methyl-D-aspartate (NMDA)-induced locomotor-like activity. A: intracellular recording from a lumbar segment 2 (L2) MN (bottom trace) and recording from the corresponding L2 ventral root (VR, top trace). Locomotor activity was induced by NMDA (7 μM) and 5-HT (7 μM). The membrane potential of the MN was recorded at varying holding potentials determined by steady current injections \( I_m \) of −400, −600, and −800 pA for segments indicated by arrows. B: part of the recording in A expanded to depict that the VR activity (top) and the MN membrane potential \( V_m \) (middle) oscillated in-phase. The L2 VR trace was rectified and smoothed. The L2 VR trace was rectified and smoothed. Dots indicate the trough, half-maximum, and peak of each cycle. Each cycle was divided into flexor phase (bins 1–5) and extensor phase (bins 6–10) with reference to half-maximum points and \( V_m \) was averaged over all calculated cycles (bottom, thin and thick traces indicate \( V_m \) of individual cycles and the average of all traces, respectively). See METHODS for details. C: extracted total \( \Delta G_{\text{total}} \), excitatory \( \Delta G_e \), and inhibitory \( \Delta G_i \) conductances. Conductances were calculated from the mean \( V_m \) traces obtained at 3 different \( I_m \) levels (top) and are indicated as a percentage of the mean \( \Delta G_{\text{total}} \) averaged over the locomotor cycle. Note that \( \Delta G_{\text{total}}, \Delta G_e, \text{ and } \Delta G_i \) show phase-dependent changes only, not including tonic components of the conductances. The excitation increased during flexor phase, whereas the inhibition varied out of phase with the excitation.
We confirmed that the net current responses could be safely regarded as linear in the voltage range recorded \((n = 6\) without dopamine; \(n = 1\) with dopamine; Fig. 2).

Excitatory and inhibitory synaptic inputs control MNs oscillations during 5-HT/NMDA–induced locomotor-like activity in an asymmetric push–pull fashion with dominant inhibitory component

We first describe the general features of conductance changes in flexor and extensor MNs during locomotor activity induced by 5-HT and NMDA without dopamine.

Figure 1 shows an example of a recording from a flexor MN in L2, with rhythmic \(V_m\) depolarizations in-phase with the L2 VR burst (Fig. 1, A and B). \(G_{\text{total}}\) varied moderately throughout the locomotor cycle, whereas the calculated \(\Delta G_e\) and \(\Delta G_i\) traces showed clear rhythmic variations (Fig. 1C). \(G_e\) increased during the flexor phase when the MN was depolarized, whereas \(G_i\) increased in the extensor phase when the MN was hyperpolarized, indicating that this flexor MN received increased excitatory synaptic drive during the flexor phase and increased inhibitory synaptic inputs during the extensor phase. As a consequence of the alternating excitation and inhibition, the total conductance was only moderately varied over the locomotor cycle.

Figure 3 shows an example of an extensor MN recorded in the L5 segment displaying rhythmic depolarizations in-phase with the main burst activity in the L5 ventral root (Fig. 3, A and B). In this extensor MN the peak-to- trough amplitude of the \(V_m\) oscillations increased with depolarization in the voltage window investigated (compare \(-70\) and \(-50\) mV; Fig. 3A). Moreover, depolarizing voltage deflections became obvious in the flexor phase when the \(V_m\) was hyperpolarized to a level below the equilibrium potential for Cl\(^-\) (arrows in Fig. 3B, bottom trace). Together these observations suggest that the \(V_m\) oscillations were dominated by rhythmic inhibitory synaptic inputs arriving in the inactive flexor phase. The extracted \(\Delta G_e\) and \(\Delta G_i\) traces clearly showed that this was the case (Fig. 3C). Although \(G_e\) and \(G_i\) showed an out-of-phase relationship with a peak of \(G_e\) in the extensor phase and a peak of \(G_i\) in the flexor phase (reciprocal to the pattern of activity seen for flexor MNs), the amplitude of \(\Delta G_e\) was only less than one fifth of that of \(\Delta G_i\). In contrast to the flexor MN described in Fig. 1, the total conductance in this extensor MN described a marked rhythmic variation, which peaked during the inactive flexor phase.

These phenotypically distinct conductance profiles described for flexor and extensor MNs shown in Figs. 1 and 3 were recapitulated in the entire population of MNs. The actual \(\Delta G\)-phase traces of all of the recorded MNs are shown in Fig. 4, A and B (top row of panels) and their normalized conductance profiles, \(\Delta G\) divided by the amplitude of the \(\Delta G\) (max \(\Delta G\)), are shown in Fig. 4, A and B (bottom row of panels). Data are divided into flexor (Fig. 4A) and extensor (Fig. 4B) MNs and data obtained in the absence and the presence of dopamine are shown in blue and red, respectively (data in the presence of dopamine are described in a following section). Before drug application the resting conductance measured from responses to small-amplitude current pulses (<200 pA, 1 s) was 22.4 \(\pm\) 2.7 nS for the L2/L3 flexor MNs, 21.0 \(\pm\) 3.2 nS for the L2/L3 extensor MNs, and 24.9 \(\pm\) 1.9 nS for L5 extensor MNs. During locomotor activity the mean \(G_{\text{total}}\) over the locomotor cycle was 17.9 \(\pm\) 1.3 nS for the L2/L3 flexor MNs, 22.7 \(\pm\) 3.2 nS for the L2/L3 extensor MNs, and 24.2 \(\pm\) 2.5 for L5 extensor MNs. The mean \(G_{\text{total}}\) during locomotion was significantly smaller than the resting conductance in L2/L3 flexor MNs (86.4 \(\pm\) 4.5\% of the resting conductance, paired \(t\)-test, \(P < 0.05\)), whereas the extensor MNs did not show significant differences between resting and locomotion, either for L2/L3 MNs nor for L5 MNs (113.2 \(\pm\) 12.5\% for L2/L3 MNs and 97.6 \(\pm\) 8.7\% for L5 MNs, \(P > 0.05\)). As can be seen from the simple average trace of normalized \(\Delta G_{\text{total}}\) (dashed line in Fig. 4A, bottom left) the flexor MNs showed little modulation of the total conductance over the locomotor cycle. However, there was a moderately lower \(G_{\text{total}}\) during their active phase (the flexor phase) than that during the inactive extensor phase. In extensor MNs, the lower conductance state during the active phase was more pronounced than that in the flexor MNs (dashed line in Fig. 4B, bottom left). The amplitude of the \(\Delta G_{\text{total}}\) was 18.4 \(\pm\) 2.4\% of the mean \(G_{\text{total}}\) value for the L2/L3 flexor MNs, 19.5 \(\pm\) 4.3\% for the L2/L3 extensor MNs, and 34.2 \(\pm\) 5.1\% for the L5 extensor MNs. The amplitude of the \(\Delta G_{\text{total}}\) in L5 extensors appeared to be larger than that in the other MNs (the difference was statistically significant between the L2/L3 flexors and the L5 extensors, Games–Howell test, \(P < 0.05\)). These results show that rodent MNs during 5-HT/NMDA–evoked locomotor-like activity have a lower conductance state during their active phase compared with that during their inactive phase.

As a whole, the excitation and inhibition varied out of phase with increased excitation in the active phase and increased inhibition in the inactive phase both in flexor MNs and extensor MNs (Fig. 4, A and B, middle and right columns, blue lines), indicating that the membrane potential oscillations of MNs are controlled by a “push–pull” synaptic drive. However, the variation of inhibition generally seemed to be larger than that of excitation (Fig. 4, A and B, top middle and top right, blue lines) and this resulted in the lower total conductance state during the active phase. This dominance of inhibition is high-
lighted in Fig. 5, where the amplitude of $\Delta G_e$ of individual MNs was plotted against the amplitude of $\Delta G_i$ (blue symbols). The amplitude of $\Delta G_i$ was in all cases larger than that of $\Delta G_e$. For L2/L3 flexor MNs the mean value of $\Delta G_e$ amplitude was 11.4 ± 1.2% of the mean $G_{total}$ and the mean value of $\Delta G_i$ amplitude was 24.8 ± 3.2% of the mean $G_{total}$. For L2/L3 extensor MNs, the corresponding values were 2.6 ± 0.6% ($\Delta G_e$) and 20.7 ± 4.4% ($\Delta G_i$) and for L5 extensor MNs values were 5.4 ± 0.7% ($\Delta G_e$) and 35.9 ± 4.9% ($\Delta G_i$). The differences between $\Delta G_e$ and $\Delta G_i$ were statistically significant in all groups (paired $t$-test, $P < 0.0002$ for the L2/L3 flexor MNs and the L5 extensor MNs, $P < 0.01$ for the L2/L3 extensors). The $\Delta G_e$ to $\Delta G_i$ ratios were 0.49 ± 0.04 for the L2/L3 flexor MNs, 0.17 ± 0.05 for the L2/L3 extensor MNs, and 0.18 ± 0.06 for the L5 extensor MNs. Thus the dominance of inhibition is more pronounced in the extensor than in the flexor MNs. These calculations clearly demonstrate an asymmetrical conductance change during the locomotor cycle with a dominance of inhibition in both flexors and extensor MNs that was extended to include both rostral and caudal MNs.

The asymmetrical conductance profiles appeared to be present at many levels of activity of the locomotor network. Thus in L2/L3 flexor MNs there was a significant correlation between the amplitudes of $\Delta G_i$ and $\Delta G_e$ ($R = 0.76$), suggesting that the two conductances at the MN level are regulated in parallel over a wide range of strength of motor outputs. For extensor MNs this correlation is not significant because $\Delta G_e$ values remain relatively constant for a large range of $\Delta G_i$. Moreover, we did not find a significant correlation between the $\Delta G$ amplitudes or the $\Delta G_e$ to $\Delta G_i$ ratio and the locomotor cycle period ($|R| < 0.521$; Fig. 6). These findings suggest that the asymmetrical conductance changes are robust phenomena during transmitter-induced locomotion and found over a large range of network outputs.

Inhibitory and excitatory conductances are tuned in a modular fashion

Inspection of the $\Delta G$ profiles shown in Fig. 4 also revealed that there seemed to be a difference in the phase tuning of $\Delta G$ curves: the profile of $\Delta G_i$ in flexor MNs and $\Delta G_e$ in extensor MNs appeared to be broader and more erratic than the $\Delta G_i$ in flexor MNs and $\Delta G_e$ in extensor MNs. To evaluate these characteristics in more detail, the preferred locomotor phase and the sharpness of the conductance-phase profile of excitation and inhibition were analyzed.

The normalized $\Delta G_e$ and $\Delta G_i$ were plotted against the locomotor phase in polar coordinates (Fig. 7, A and B, top rows, blue lines); we then calculated the mean vector for each MN (Fig. 7, A and B, bottom rows, blue symbols). The angle of the mean vector represents the preferred active phase and the length represents the sharpness of $\Delta G$-locomotor-phase profile, for each conductance. The preferred locomotor phases of $\Delta G_e$ and $\Delta G_i$ are plotted for flexor MNs and extensor MNs in Fig. 7.
7C. This plot highlights the timing of the conductance changes as described earlier. In all of the flexor MNs and most (13/15) of the extensor MNs, the preferred phase of conductance change clearly alternated with increased excitation in the active phase and increased inhibition in the inactive phase. Only two L5 extensor MNs showed a concurrent increase of excitation and inhibition with the peaks in the inactive flexor phase. Thus the general feature of the conductance change is excitation alternating with inhibition.

The average vector length for $\Delta G_e$ was significantly longer than that for $\Delta G_i$ in L2/L3 flexor MNs (0.53 ± 0.01 vs. 0.40 ± 0.03, paired t-test, $P < 0.0001$; Fig. 7D). In contrast, the vector length for $\Delta G_e$ in L2/L3 extensor MNs was significantly shorter than that for $\Delta G_i$ (0.29 ± 0.05 vs. 0.49 ± 0.04, paired t-test, $P < 0.05$; Fig. 7D). These results indicate that in L2/L3 the excitation in flexor MNs and the inhibition in extensor MNs, which increase timewise simultaneously during the flexor phase, had a sharper conductance-phase profile than that of conductances that increased during the extensor phase. On the other hand, $\Delta G_e$ and $\Delta G_i$ in L5 extensors had similar average vector lengths (0.48 ± 0.04 vs. 0.56 ± 0.03, paired t-test, $P > 0.1$; Fig. 7D). There was also a significant difference between $\Delta G_e$ values in extensors in L2/L3 and L5 (unpaired t-test, $P < 0.05$). These findings suggest that conductances in flexor and extensor MNs in L2/L3 are modulated in a modular fashion and different from those in L5.

Asymmetrical conductance profiles are preserved in the presence of dopamine

One possibility for the observed asymmetrical conductance changes is that 5-HT and NMDA bias the network operation in a skewed way, in particular because 5-HT has been reported to have stronger excitatory effects on extensor- than on flexor-related motor circuits in the mammalian spinal cord (see Conway et al. 1988; Schmidt and Jordan 2000; Vult von Steyern and Lømo 2005). To test whether similar profiles are observed when locomotor-like activity is produced by drug

FIG. 4. Locomotor-phase relationships of conductance change. A: $\Delta G_{total}$ (left column), $\Delta G_e$ (middle column), and $\Delta G_i$ (right column) in flexor MNs ($n =$ 19 without dopamine, blue lines; $n =$ 3 with dopamine, red lines) plotted against locomotor phase. Actual values (percentage of mean $G_{total}$) are shown in the top row and values normalized by the maximum amplitude (max $\Delta G$) are shown in the bottom row. Dashed lines in the normalized graphs are the average of all traces. B: data for extensor MNs ($n =$ 15 without dopamine; $n =$ 3 with dopamine), arranged as in A. Concentrations of drugs: NMDA (5–10 $\mu$M), 5-HT (5–10 $\mu$M), dopamine (25–50 $\mu$M).
combinations that may bias the CPG circuits different from 5-HT we used a cocktail of 5-HT/NMDA in combination with dopamine (25–50 μM) in a small number of experiments (n = 3 flexor MNs in L2; n = 3 extensor MNs in L5) to evoke rhythmic activity. This cocktail is commonly used to induce locomotor-like activity in the rodent (Jiang et al. 1999) and 5-HT and adrenergic modulation is also known to have opposite effects on inhibition in vertebrate CPGs (McDearmid et al. 1997; McLean et al. 2000).

The MNs showed profiles of excitation and inhibition parallel with those in the absence of dopamine. The excitation and inhibition varied out of phase with each other with increased excitation during the active phase and increased inhibition during the inactive phase in all MNs (Figs. 4 and 7C, red lines). The amplitude of ΔGe (39.4 ± 10.4% of mean Gtotal for L2 flexors MNs; 32.2 ± 2.8% for L5 extensor MNs) was larger than that of ΔGi (15.1 ± 3.1% for L2 flexor MNs; ΔGe/ΔGi ratios: 0.40 ± 0.04 for L2 flexor MNs, 0.20 ± 0.5 for L5 extensor MNs) in all MNs and the dominance of inhibition was most pronounced in extensor MNs in L5 extensor MNs in L5 extensor MNs) in all MNs and the dominance of inhibition was most pronounced in extensor MNs (ΔGe to ΔGi ratios: 0.40 ± 0.04 for L2 flexor MNs, 0.20 ± 0.5 for L5 extensor MNs; Fig. 5, red symbols). Similarly, there were no obvious differences in the phase tunings [mean vector length: 0.55 ± 0.01 (ΔGe) and 0.41 ± 0.05 (ΔGi) for L2 flexor MNs; 0.50 ± 0.03 (ΔGe) and 0.56 ± 0.07 (ΔGi) for L5 extensor MNs] from those in the absence of dopamine (Fig. 7, A and B, red). These results suggest that the asymmetrical conductance changes have sharper locomotor phase tuning than that of the

**DISCUSSION**

The present study provides a quantitative assessment of the synaptic conductance impinging onto lumbar MNs in the neonatal mouse spinal cord during drug-induced locomotor-like network activity. In a large majority of MNs the excitatory and inhibitory conductance varied reciprocally, but in an asymmetric manner where inhibition was dominating over excitation, especially in extensor MNs. The asymmetrical conductance profile appears to be a hallmark of the transmitted synaptic signaling because it applies to a range of locomotor outputs and different experimental locomotor conditions. Analysis of the phase tuning of the excitatory and inhibitor conductances in flexor and extensor MNs in the upper lumbar cord showed that the flexor-phase–related synaptic conductance changes have sharper locomotor phase tuning than that of the

![Figure 5](http://jn.physiology.org/)

**FIG. 5.** Relative weight of excitatory and inhibitory conductances. Amplitude of ΔGe is plotted against that of ΔGi for each MN. Dashed line indicates diagonal (ΔGe = ΔGi) line. Modulation of inhibitory conductance dominates over that of excitatory conductance in all of the recorded MNs. Blue symbols indicate data in the absence of dopamine and red symbols indicate data in the presence of dopamine. Open circles indicate flexor MNs in L2/L3, filled circles indicate extensor MNs in L2/L3, and crosses indicate extensor MNs in L5.

![Figure 6](http://jn.physiology.org/)

**FIG. 6.** Relationships between the change of synaptic conductances and locomotor cycle period. The amplitude of ΔGe (A) and ΔGi (B) and the ratio between the amplitude of ΔGe and ΔGi (C) are plotted against the mean locomotor cycle period during the recording for each MNs. None of these parameters shows clear dependence on the cycle period. Blue symbols indicate data in the absence of dopamine and red symbols indicate data in the presence of dopamine. Open circles indicate flexor MNs in L2/L3, filled circles indicate extensor MNs in L2/L3, and crosses indicate extensor MNs in L5.
extensor-phase–related conductances, suggesting differential control/composition of flexor and extensor modules in this part of the cord. Our analysis furthermore showed that phase tunings of the conductance changes in rostral and caudal MNs were different, suggesting differential control of MN activity in these anatomically separated parts of the cord. The asymmetric nature and phase tuning of the conductance profiles provide important clues to the organization of the rodent locomotor CPG and predict a multilayered structure.

Methodological considerations

The present conductance measurement and decomposition method has previously been used to evaluate the network activity converging onto identified neuron classes in the brain and spinal cord (Anderson et al. 2000; Berg et al. 2007; Borg-Graham et al. 1998; Marino et al. 2005; Shu et al. 2003). The general assumption is that the total conductance consists of excitatory and inhibitory synaptic conductances and a leak conductance, which includes all other conductances. The basic requirement is that no active conductances are activated during the voltage oscillations. In the present study, we have attempted to fulfill this requirement in two ways. First, voltage-dependent potassium and sodium conductances, including the persistent sodium conductance that is known to be expressed in rodent MNs (Kuo et al. 2006; Li and Bennett 2003), were reduced by intracellular Cs/H11001 and QX-314, respectively. Second, recordings were performed at relatively hyperpolarized levels to avoid influences from voltage-dependent conductances including NMDA receptors. Under these conditions, we confirmed that the current–voltage relationship was linear in the presence of locomotion-inducing drugs in the voltage range where the measurements were performed. This also indicated that subthreshold conductances like the transient calcium conductance or hyperpolarization-activated inward cation conduc-

![Figure 7: Preferred phase and phase tuning of excitatory and inhibitory conductances. A and B: normalized ∆G value (top rows) and the endpoint of the mean vector (bottom rows) of ∆G_e (left columns) and ∆G_i (right columns) for each flexor (A) and extensor (B) MN plotted against locomotor phase in polar coordinates. The angle of the mean vector indicates preferred locomotor phase and the length indicates the sharpness of the phase tuning of the conductance. The radius axis ranges from 0 to 1 and the dotted circle indicates 0.5. Blue and red indicate data in the absence and the presence of dopamine, respectively. Open circles indicate MNs in L2/L3 and crosses indicate MNs in L5. C: the preferred locomotor phase for the excitation and inhibition in each MN. The color code is the same as that in A and B. D: in L2/L3 MNs the length of the mean vector is longer for the excitation in flexor MNs and the inhibition in extensor MNs than for the inhibition in flexor MNs and excitation in extensor MNs. The differences are not seen in L5 extensor MNs. Error bars indicate SE.]

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tance that are known to be present in rodent motor neurons and to be facilitated by 5-HT (Berger and Takahashi 1990; Kjaer-ullff and Kiehn 2001; Larkman and Kelly 1997) did not cause a significant nonlinearity. Moreover, as previously demonstrated $I_h$ will mainly contribute with a leak conductance during locomotion and thus will not be modulated in a time-varying way (Kiehn et al. 2000).

We had to set unknown parameters that appear in Eqs. 1 and 2 (see METHODS). We could determine realistic values for the reversal potentials of excitatory and inhibitory synaptic conductances. However, it was impossible to isolate and measure the leak conductance and its reversal potential during the network activity in our experimental conditions (see also Berg et al. 2007). These limitations thus did not allow us to calculate the actual values of the synaptic conductances. Instead, we calculated only the locomotor-phase–related changes of the excitation and inhibition: $\Delta G_e$ and $\Delta G_i$, respectively. $\Delta G_e$ and $\Delta G_i$ do not depend on the precise estimation of the leak-related conductances as long as these are constant throughout the measurement (cf. Berg et al. 2007). Although some voltage-dependent conductances might be enhanced or depressed during the network activity, we believe that these conductances were not significant factors in the present experimental conditions as discussed earlier.

Another possible source of error is the cable properties of MNs. It is possible that a relatively distant location from soma of the excitatory synaptic inputs compared with inhibitory synaptic inputs caused the smaller relative amplitude of $\Delta G_e$ than $\Delta G_i$. Nevertheless, the present results obviously reflect a relative impact of the excitation and inhibition on the somatic membrane potential and the output of the MNs.

Activity of motor neurons is regulated by alternating synaptic inputs in a push–pull manner with dominant inhibitory conductance

The synaptic conductance profiles extracted from the rhythmic signals recorded during drug-induced locomotor activity showed in the large majority of MNs alternation between excitation and inhibition. Excitatory conductance peaked in the depolarizing phase, whereas the inhibitory conductance peaked in the hyperpolarizing phase (push–pull). These results confirm a large number of previous in vivo and in vitro studies in the vertebrate spinal cord on rhythmic MN oscillations during walking (Burke et al. 2001; Cazalets et al. 1996; Hochman and Schmidt 1998; Jordan 1983; Orsal et al. 1986; Schmidt 1994; Schmidt et al. 1986; Shefchyk and Jordan 1985a,b), swimming (Kahn 1982; Russell and Wallen 1983; Wallen et al. 1993), and scratching (Perreault 2002; Robertson and Stein 1988). The exception to this general notion of a push–pull organization is a series of recent studies by Houngsaard and colleagues, which demonstrated a balanced variation of opposing synaptic conductances during the depolarizing phase in hindlimb MNs recorded during scratching movements (Alaburda et al. 2005; Berg et al. 2007). The balanced excitation and inhibition lead to a remarkably high total conductance state during the active phase of MN firing. In the present study, we find signs of balanced variation of opposing synaptic conductances only in two MNs. Moreover, in accordance with previous reports (Burke et al. 2001; Cazalets et al. 1996; Jordan 1983, 1991; Perreault 2002; Schmidt 1994; Schmidt et al. 1986; Shefchyk and Jordan 1985b), total conductance was generally unchanged or moderately lower in the hyperpolarizing phase in the present experiments. We do not have a clear explanation for the discrepancies between the in vitro turtle scratch experiments and other experiments. One possible explanation is that recordings in the turtle were performed from MNs located near the transversely cut end of the cord and that some parts of the pre-MN network are therefore missing.

The synaptic conductance measurements allowed a direct quantitative analysis of time-varying synaptic conductances and in particular to investigate whether the conductance profiles were equal in flexors and extensors. Previous studies have suggested a predominance of inhibitory drive to some mammalian MNs during locomotion (Hochman and Schmidt 1998; Schmidt et al. 1988; Shefchyk and Jordan 1985a). Here, we find that in all MNs the time-varying conductance changes were asymmetric in amplitude, with inhibition dominating over excitation. Furthermore, this inhibitory dominance was largest in extensors (both L2/L3 and L5) where the average excitatory conductance changes range to only 15–20% of the inhibitory conductance changes. In flexors $\Delta G_i$ was about twice the amplitude of $\Delta G_e$. Our findings thus show that last-order rhythmic excitatory inputs from the CPG network to MNs are relatively small compared with inhibition and suggest that rhythmicity of extensor MNs is more susceptible to changes in central excitatory drive than flexor MNs. Some indications of this come from findings in cats showing that spontaneous failures of burst of activity in MN firing—so-called deletions—occur more frequently in extensor than in flexor MN pools (Duyssens 1977; Lafreniere-Roula and McCrea 2005). Such deletions are rare when sensory input is intact, possibly because a greater part of the amplitude modulation in extensor MNs comes from extensor group I afferent feedback, whereas flexor-directed feedback is more variable (Hiebert and Pearson 1999; Hultborn 2001; McCrea 2001; Nielsen and Sinkjaer 2002; Pearson 2004). The predominance of inhibition also suggests that mammalian MN rhythmicity is regulated around a tonic excitation that will increase the importance of the tonic inhibitory signals (see Jordan 1983; Roberts and Tunstall 1990).

Tuning of synaptic conductances reveal modular organization of CPG with tighter rhythm-generating capabilities in flexor modules

The analysis of the phase tuning of the conductance changes in L2/L3 flexor and extensor MNs showed a similarity in the sharpness of phase tuning between excitatory and inhibitory synaptic drives which increased in phase (e.g., excitation of flexors MNs and inhibition of extensor MNs), and a significant difference in phase tuning between synaptic drives, which increased out of phase. These observations support the notion that pre-MN excitatory and inhibitory interneuronal circuits work as cooperating modules/units (see Jordan 1991; Stein 2005), which convey reciprocal activities to flexor and extensor MNs with a common fingerprint.

In contrast to L2/L3, the difference in the phase tuning in L5 extensor MNs was not obvious and the tuning of excitation L5 extensor MNs was different from that of L2/L3 extensor MNs, indicating that MNs in rostral and caudal lumbar segments are under the control of functionally distinct CPG networks, re-
spectively (see also Tresch and Kiehn 1999). In the mouse, MNs located in L5 may belong to the intrinsic foot muscles, whereas MNs located in L2/L3 innervate the hip and knee (McHanwell and Biscoe 1981). Our results are thus compatible with results from the cat where it has been shown that MNs innervating the intrinsic foot muscles, located in caudal lumbar segments, receive different central drive than rostrally located extensor MNs (Burke et al. 2001). In future studies it will obviously be of great interest to be able to perform the conductance measurements in muscle-identified groups of MNs.

The extensor-related synaptic drives (excitation of extensors MNs and inhibition of flexor MNs) were in all cases loosely tuned to the locomotor phase and/or having weaker rhythmic modulation, compared with flexor-related drives (excitation of flexor MNs and inhibition of extensor MNs) that were more sharply tuned and displayed larger rhythmic modulations. These and previous observations (see e.g., Duysens 1977; Lafreniere-Roula and McCrea 2005) imply that the flexor-related premotor CPG network operates more robustly and stably with tighter rhythm-generating capabilities compared with the extensor-related CPG modules. The flexor-related network modules may therefore have a leading role in the operation of locomotor CPG in the spinal cord. However, our observations do not imply that the extensor parts of the CPG are collaterals to rhythmic flexor modules (Brownstone and Wilson 2008; Pearson and Duysens 1976) and are incapable of generating rhythmic activity. In fact, the push–pull nature of the synaptic inputs observed in extensor MNs exclude the pure flexor-generator model (Pearson and Duysens 1976; see also the discussion in Shefchyk and Jordan 1985) and the observed differences in drive between L2/L3 and L5 extensor MNs also preclude an asymmetric model with a rhythmogenic flexor module localized in the L1–L2 region, as proposed by Brownstone and Wilson (2008).

The asymmetries in CPG operation were observed when we used the two classical drug cocktails to induce rhythmic locomotor activity in rodents (Jiang et al. 1999): 5-HT in combination with NMDA or 5-HT/NMDA combined with dopamine. 5-HT is known to have a stronger excitatory effect on extensor MNs as well. These latter observations indicate that the “gain” in the last-order excitatory and inhibitory interneuron pathways (e.g., those controlled by the flexor module/unit) can be controlled independently. This independent gain control may be explained by an organization where the last-order inhibitory interneurons are not downstream collaterals of excitatory last-order interneurons, as proposed in most models of the mammalian CPG models (Brownstone and Wilson 2008; Jordan 1991; Kriellaars et al. 1994; Lafreniere-Roula and McCrea 2005; Noga et al. 2003; Perret et al. 1988; however, see Burke et al. 2001). The pure reciprocal coupling at the last-order interneuron level proposed in previous models is presumably based on findings showing that in some cases of MN recordings a single synapse segmental delay can be observed between excitatory and inhibitory synaptic potentials following stimulation of the mesencephalic locomotor region (MLR) (Jordan 1991; Noga et al. 2003; Shefchyk and Jordan 1985a). However, in many cases of MLR stimulation this strict single-synapse excitatory–inhibitory coupling is replaced by

**Consequences for organization of the walking CPG**

The findings that the rhythmic last-order inputs to MNs are asymmetric with a dominance of inhibition are compatible with an organization where the number of last-order excitatory interneurons is considerably smaller than the number of last-order inhibitory interneurons both in the flexor and extensor modules/units. Moreover, we find no correlation between the amplitudes of $\Delta G_e$ and $\Delta G_i$ in extensor MNs and, although there was a correlation between $\Delta G_e$ and $\Delta G_i$ in flexor MNs, $\Delta G_e$ amplitude seemed to saturate for larger values of $\Delta G_i$ in flexor MNs as well. These latter observations indicate that the “gain” in the last-order excitatory and inhibitory interneuron pathways (e.g., those controlled by the flexor module/unit) can be controlled independently. This independent gain control may be explained by an organization where the last-order inhibitory interneurons are not downstream collaterals of excitatory last-order interneurons, as proposed in most models of the mammalian CPG models (Brownstone and Wilson 2008; Jordan 1991; Kriellaars et al. 1994; Lafreniere-Roula and McCrea 2005; Noga et al. 2003; Perret et al. 1988; however, see Burke et al. 2001). The pure reciprocal coupling at the last-order interneuron level proposed in previous models is presumably based on findings showing that in some cases of MN recordings a single synapse segmental delay can be observed between excitatory and inhibitory synaptic potentials following stimulation of the mesencephalic locomotor region (MLR) (Jordan 1991; Noga et al. 2003; Shefchyk and Jordan 1985a). However, in many cases of MLR stimulation this strict single-synapse excitatory–inhibitory coupling is replaced by

![Diagram](http://jn.physiology.org/)

**FIG. 8.** Schematic diagram of the proposed organization of the rodent locomotor central pattern generator (CPG). The asymmetrical nature of the reciprocal synaptic conductances with inhibitory dominance is indicated as a difference in the number of last-order excitatory (red) and inhibitory (blue) interneurons projecting onto flexor and extensor MNs. These 2 sets of last-order interneurons are under common control of flexor- or extensor-related excitatory neurons that may or may not have rhythm-generating properties. The flexor-related network operates more robustly and stably with tighter rhythm-generating capabilities than the extensor-related network (as indicated with the thicker curved arrow). A further upstream rhythm-generating layer reciprocally connecting flexor and extensor modules is indicated with question marks. We found no direct evidence for this layer in the present study, but suggest that it may be included in the CPG organization to most easily explain the so-called nonresetting deletions reported in previous locomotor studies (see text for further details).
overlapping excitatory and inhibitory latencies or by pure excitatory or inhibitory inputs (Degtyarenko et al. 1998; Noga et al. 2003). To encompass the strict single-synapse excitatory–inhibitory coupling with independent gain control at the last-order level, we propose a model with populations of excitatory and inhibitory last-order interneurons driven by common upstream excitatory interneurons, which themselves also directly project onto MNs. Our findings thus support the notion of a multilayer structure of the mammalian CPG consisting of at least two layers (Fig. 8), with the important addition of a skewed number of last-order inhibitory and excitatory interneuron and possibilities for increasing the inhibitory conductance in the face of a constant excitation.

Based on the conductance measurements, we cannot determine whether the second layer in our model has rhythm-generating capability by itself and/or is driven by further upstream rhythm-generating neurons. However, by implementing further upstream rhythm-generating layers in the flexor and extensor modules, the so-called nonresetting deletions, which are commonly seen as loss of activity in flexor or extensor MNs accompanied by close to tonic activity in antagonist MNs, can more easily be explained in the model (Burke et al. 2001; Kriellaars et al. 1994; Laffreniere-Roula and McCrea 2005). Although not shown in the figure our data also suggest that the locomotor CPG is composed of several flexor/extensor modules/units in the rostral and caudal lumbar spinal cord (see also Laffreniere-Roula and McCrea 2005; McCrea and Rybak 2008).

Conclusion

The present quantitative conductance analysis in rodent motor neurons clearly shows that the structure and/or operation of flexor-related and extensor-related CPG modules of locomotion are reciprocally but asymmetrically organized. The data predict a layered structure of the rodent CPG with a skewed distribution of last-order inhibitory and excitatory interneurons placed under common control of excitatory second last-order neurons. The overall structure of the proposed model resembles the multilayered modular architecture proposed for locomotor CPGs in felines. The predictions of the model provide a solid basis for further anatomical and electrophysiological investigations of the cellular organization of the mammalian CPG.

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