Optogenetic dissection reveals multiple rhythmogenic modules underlying locomotion

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Neural networks in the spinal cord known as central pattern generators produce the sequential activation of muscles needed for locomotion. The overall locomotor network architectures in limbed vertebrates have been much debated, and no consensus exists as to how they are structured. Here, we use optogenetics to dissect the excitatory and inhibitory neuronal populations and probe the organization of the mammalian central pattern generator. We find that locomotor-like rhythmic bursting can be induced unilaterally or independently in flexor or extensor networks. Furthermore, we show that individual flexor motor neuron pools can be recruited into bursting without any activity in other nearby flexor motor neuron pools. Our experiments differentiate among several proposed models for rhythm generation in the vertebrates and show that the basic structure underlying the locomotor network has a distributed organization with many intrinsically rhythmogenic modules.

channelrhodopsin-2 | halorhodopsin | motor neurons | interneurons

Spinal cord networks that drive and coordinate walking are, to a large extent, innate. Even in species that do not walk at birth, such as rodents and humans, the networks that generate walking are already present (1, 2). The early development of functional locomotor networks has been exploited and studied in the neonatal rodent spinal cord in vitro preparation in which locomotor-like activity can be induced pharmacologically or by electrical activation of descending or afferent fibers (3–6). The mammalian locomotor networks have also been studied by using the adult cat where locomotor-like activity similarly can be induced pharmacologically or electrically (7–9). A general notion from these studies is that forelimb and hindlimb locomotion are controlled by independent limb-controlling circuits (10) and that rhythm-generating excitatory neurons, and pattern-generating neurons, interact to produce the coordinated motor output (11–14). The network layout within these limb-controlling circuits has, however, been debated. A number of conceptual models have been advanced. The classical half-center model asserts that flexor and extensor bursting is generated by two reciprocally coupled half-centers driving all flexors and extensors (8, 15). The flexor burst generator model is asymmetric and consists of a flexor burst generator that provides active excitation of flexor motor neurons and inhibition of extensor motor neurons that are otherwise tonically active (14, 16, 17). In response to evidence that the central pattern generator (CPG) could produce a more complex motor output than just a mere flexor-extensor alternation (9), the unit burst generator (UBG) model was proposed (18). According to this theory, separate modules can generate a rhythm in close muscle synergies and are distributed around each joint (18, 19), or in the swimming network in each hemi-segment (20). The UBGs therefore generate a local rhythmic activity that during locomotion will be recruited so that they form an interconnected network of rhythm generators. The conceptual models for the overall locomotor network architecture have been deduced from microlesion studies (21–24), pharmacological activation of restricted areas of the cord (25, 26), afferent perturbation studies (27–29), and deletion studies (14, 16, 30). More recently, genetic ablation studies have also added to the

understanding of details of the organizational structure of the rodent locomotor network (31).

In this study, we use an optogenetic approach in which spinal excitatory and inhibitory interneurons are genetically targeted for selective activation or inhibition to probe the overall organization of the spinal hindlimb locomotor network in mice. We first show that spinal glutamatergic neurons are both sufficient and necessary for generating rhythmic activity. Activating smaller populations of glutamatergic neurons in the cord limits the rhythmic output to restricted flexor-related or extensor-related motor neurons. Our experiments demonstrate a modular organization of the hindlimb locomotor network where multiple rhythmogenic flexor and extensor modules are combined to produce an integrated motor output.

Results

Glutamatergic Neurons Are Sufficient and Necessary for Generating Locomotor-Like Activity. We have shown earlier that tonic light-induced activation of a ventral population of excitatory interneurons in the rostral lumbar spinal cord can elicit locomotor-like activity in the hindlimb motor neuron pools (32). To further assess the role of the spinal interneurons in generating the locomotor rhythm, we used a conditional approach that allowed us to activate or inhibit spatially segregated populations of excitatory or inhibitory neurons in the spinal cord.

To activate the excitatory population of neurons in the spinal cord, we crossed *Vesicular glutamate transporter 2* (*Vglut2*)::causes recombination (*Cre*) with *Rosa26*-cytomegalovirus early enhancer element/chicken beta-actin promoter (*CAG*)-*lox*P–Stop codons–3x SV40 polyA–*lox*P (*LSL*)-channelrhodopsin2 (*ChR2*)-enhanced yellow fluorescent protein (*EYFP*)-woodchuck hepatitis virus posttranscriptional enhancer (*WPRE*) (RC-ChR2) mice (33). Vglut2 is the dominant glutamatergic transporter in interneurons in the spinal cord, and *Vglut2::Cre* mice have expression specifically restricted to all glutamatergic cells (34). YFP-positive neurons recorded in these mice show strong responses to light with persistent spiking when all excitatory fast synaptic transmission is blocked by 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) and D-(-)-2-amino-5-phosphonopentanoic acid (AP5) (Fig. 1A), demonstrating that these neurons respond directly to the light stimulus. To monitor locomotor activity, the output of ventral roots from lumbar 2 (L2) and lumbar 5 (L5) segments on the left (lL2, lL5) and right sides (rL2, rL5) were recorded. When blue light was shined onto the ventral surface of the lumbar cord (L1–L6), locomotor-like activity was evoked, characterized by an alternation in bursts from ipsilateral flexor-dominant L2 and extensor-dominant L5 ventral roots and ipsilateral and contralateral L2 (or L5) roots, respectively (Fig. 1C). Instantaneously light-evoked locomotor-like activity was seen in all preparations,

Fig. 1 (left column)

A Vglut2::Cre x RC-ChR2
APV + CNQX
-50
20mV
2s

B Vglut2::Cre x RC-eNpHR
5mV
1s

C Vglut2::Cre x RC-ChR2
Lumbar light stimulation
rL2
lL2
lL5
2s

flexor/extensor   left/right
Frequency (Hz)
4
2
1
0.5
0.25
270 180 0 90   270 180 0 90
0.9
0.8
0.7
lL2
lL5   lL2
rL2
5s

D Vglut2::Cre x RC-eNpHR
NMDA and 5HT
lL2
lL5
rL5
5s

E VIAAT::Cre x RC-ChR2
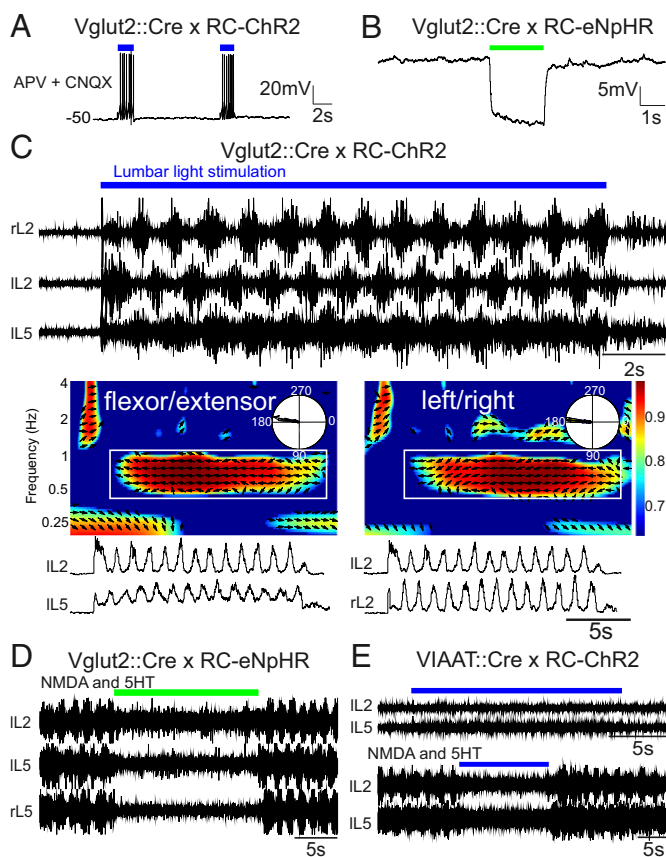lL2
lL5
5s
NMDA and 5HT
lL2
lL5
5s

Fig. 1. Glutamatergic neurons are both sufficient and necessary for evoking locomotor-like activity, whereas inhibitory neurons can only abolish ongoing locomotor activity. Light depolarizes *Vglut2::Cre; RC-ChR2*-expressing neurons despite blockade of glutamatergic neurotransmission (AP5 and CNQX) (*A*) and hyperpolarizes *Vglut2::Cre; RC-eNpHR*-expressing neurons (*B*). (*C*) Stimulating the ventral surface of the lumbar spinal cord of *Vglut2::Cre; RC-ChR2* mice evokes locomotor-like activity with direct onset and well-tuned flexor-extensor and left-right coordination. The time-frequency plot shows the coordination between flexor-extensor (*Middle Left*) and left-right (*Middle Right*) activity. The color coding denotes the coherence between the two signals and the vector field shows phase delay (also indicated in the circular plots, right = 0°, down = 90°) (*Bottom*). (*D*) Inhibiting glutamatergic neurons in the *Vglut2::Cre; RC-eNpHR* mouse turns off ongoing drug-evoked (5-HT/NMDA: 7 μM/8 μM) activity. Activating inhibitory interneurons does not elicit any rhythmic activity (*E*) but turns off ongoing drug-evoked (5-HT/NMDA: 7 μM/8 μM) activity.

in absence of any other locomotor-stimulating activity (*n* = 14, average frequency = 1.06 Hz, range 0.40–2.70 Hz, flexor-extensor coordination *r* value = 0.90, left-right *r* value = 0.83). In contrast to what was seen in previous experiments using the *Vglut2-ChR2* mouse (32), the locomotor activity persisted for a longer time, essentially as long as the light stimulus was on. The patterning between flexor-extensor and left-right activity was finely tuned, as seen in the time-frequency and circular plot from the locomotor period (Fig. 1*C*). The color coding denotes the coherence of the two signals and the vector field shows the phase relationship between flexor-extensor and left-right signals, respectively. These experiments demonstrate the sufficiency of Vglut2-positive cells for generating hindlimb locomotion.

To probe the necessity of glutamatergic neurons for locomotion, we inhibited Vglut2-positive neurons during ongoing locomotor-like activity induced by the locomotor-evoking drugs serotonin (5-HT)/NMDA, brainstem stimulation (BS), or dorsal root stimulation. For these experiments, we used *Vglut2::Cre* crossed with *Rosa26-CAG-LSL*-enhanced natronomonas pharaonis halorhoposin (*eNpHR*)-*EYFP-WPRE* (*RC-eNpHR*) mice. Intracellular

Right column

recordings of excitatory cells show that shining light onto them caused a persistent and strong hyperpolarization (Fig. 1*B*). When light was shined onto the *en bloc* spinal cord during locomotor-like activity induced by descending (*n* = 9), dorsal root (*n* = 3), or pharmacological activation [N-methyl-D-aspartate (NMDA) and 5-hydroxytryptamine (5-HT), *n* = 4] (Fig. 1*D*), a complete inhibition of the ongoing rhythm was seen.

As a counterpart to manipulating the glutamatergic population of cells, we performed light stimulation of inhibitory neurons in the spinal cord, identified by their expression of the vesicular inhibitory amino acid transporter (VIAAT) (35). To target these cells, we used a transgenic *VIAAT::Cre* mouse (Fig. S1). This mouse strain expresses Cre exclusively in GABAergic and glycinergic cells in the spinal cord (98.7% recombination in glycine and GABA-positive cells; *SI Methods*). The *VIAAT::Cre* mice were crossed with *RC-ChR2* mice. Light stimulation of the spinal cord of *VIAAT::Cre; RC-ChR2* mice does not evoke any kind of rhythmic activity (*n* = 3) (Fig. 1*E*, *Upper*). Conversely, during drug-evoked (5-HT/NMDA) locomotor-like activity, tonic light stimulation of the entire lumbar spinal cord in *VIAAT:: Cre; RC-ChR2* (Fig. 1*E*, *Lower*) mice caused a strong inhibition of ventral root output.

Together, these experiments show that Vglut2-positive cells are both sufficient and necessary for rhythm-generation and locomotion, whereas activation of VIAAT-positive cells cannot initiate locomotion but can inhibit ongoing bursting.

A Locomotor-Like Rhythm Can Be Initiated from both Rostral and Caudal Parts of the Spinal Cord. To probe the distribution of the locomotor network, we light-stimulated different areas of the lumbar spinal cord in *Vglut2::Cre; RC-ChR2* mice. A rhythmic output with left-right and flexor-extensor alternation could be evoked when the rostral lumbar spinal cord (L1–L3) was stimulated with a light spot centered at the L2 segment covering an area of 0.4–2.5 mm² (Fig. 2*A*). A similar result was seen when the caudal lumbar (L4–L6) spinal cord was stimulated (Fig. 2*B*). The light threshold for evoking coordinated locomotor-like output was variable across different animals, but always lower in the rostral lumbar spinal cord compared with the caudal lumbar spinal cord. Moreover the frequency of the evoked locomotor-like activity was lower when evoked by stimulating the caudal compared with the rostral lumbar spinal cord (0.73 Hz and 1.14 Hz, respectively; *P* = 0.014, *n* = 9). This rostrocaudal

Fig. 2

A Vglut2::Cre x RC-ChR2
L1
L2
L3
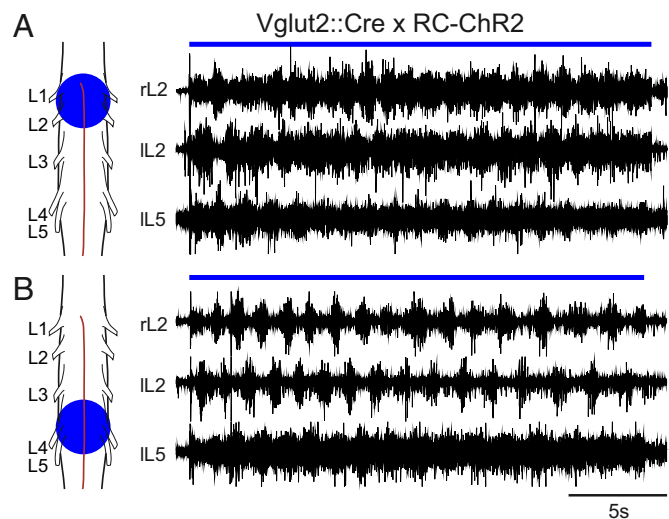L4
L5
rL2
lL2
lL5

B
L1
L2
L3
L4
L5
rL2
lL2
lL5
5s

Fig. 2. Locomotor-like activity can be elicited from rostral or caudal regions. Smaller stimulation areas can evoke locomotor-like activity. Spots ranging from 0.4–2.5 mm² could successfully evoke locomotor-like activity when centered over rostral (*A*) or caudal (*B*) lumbar spinal cord.

gradient in rhythmogenic potential corresponds to what has been observed when the locomotor network was activated with neuroactive substances (23, 25, 36)

Locomotor Activity Can Be Unilaterally Induced. Having established that the locomotor-like activity can be induced by stimulating large areas of the cord in *Vglut2::Cre; RC-ChR2* mice, we aimed to activate more restricted areas to test whether rhythmic activity could be induced in isolation. Unilateral activity could be induced by stimulating one side of the cord ($n = 6$, mean frequency = 1.00 Hz, range 0.69–1.49 Hz). An example is shown in Fig. 3A where an area of interest was stimulated consecutively on first the left, then the right, side of the cord in L2. Persistent rhythmic activity was initiated on the left side with no rhythmic activity on the right when the left side was stimulated and vice versa. The change of rhythmic activity was instantaneous when the stimulation side was switched. The rhythmogenic capability on one side did not depend on bilateral shared networks (37), because rhythmic activity could also be induced on one side of the cord when the ventral commissure was cut from the upper thoracic to the sacral spinal cord ($n = 3$) (Fig. 3B).

These experiments support the notion that a hemicord can generate a rhythmic motor output, which has also been demonstrated pharmacologically (38, 39). This concept was further supported by experiments in the *Vglut2::Cre; RC-eNpHR2* mice where light applied to one hemicord could shut down brainstem-evoked (BS) locomotor-like activity on one side of the cord with activity persisting on other side of the cord without any clear change in frequency (paired *t* test $P = 0.38$, $n = 7$) on the unstimulated side (Fig. 3C).

Rhythmic Bursting Can Be Induced or Persist Independently in Flexor and Extensor-Related Motor Neuron Pools. The possibility to stimulate restricted areas with light allowed us to ask two additional questions about the rhythm-generating capability: (*i*) can flexor
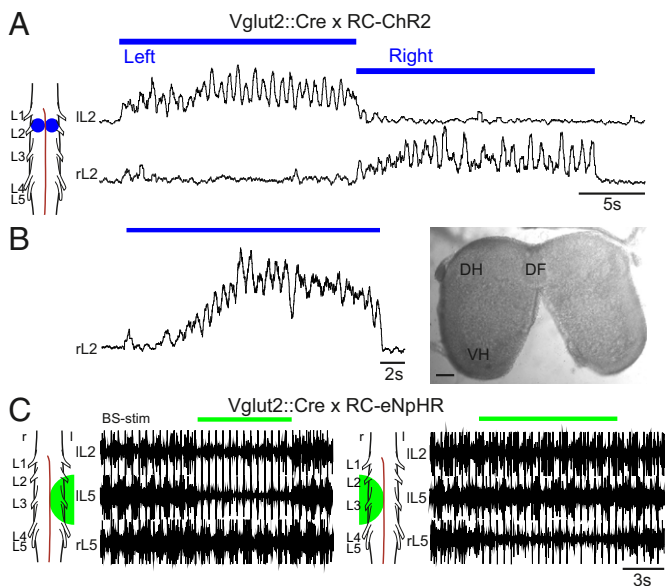
Fig. 3. Locomotor-like activity can be initiated and eliminated unilaterally. Stimulating a unilateral spot at L2 in *Vglut2::Cre; RC-ChR2* mice elicits unilateral locomotor-like activity in left and right sides consecutively (*A*), and this activity can be produced when the two sides are separated by cutting the ventral commissure (*B*; DF, dorsal funiculus; DH, dorsal horn; VH, ventral horn). (Scale bar: 100 μm.) Hyperpolarizing glutamatergic neurons unilaterally, using *Vglut2::Cre; RC-eNpHR* mice can abolish brainstem-evoked (BS-stim: 2 Hz, 0.5 mA) locomotor-like activity on one side of the cord, whereas activity is maintained on the side contralateral to the light (*C*; l and r, left and right sides, respectively).
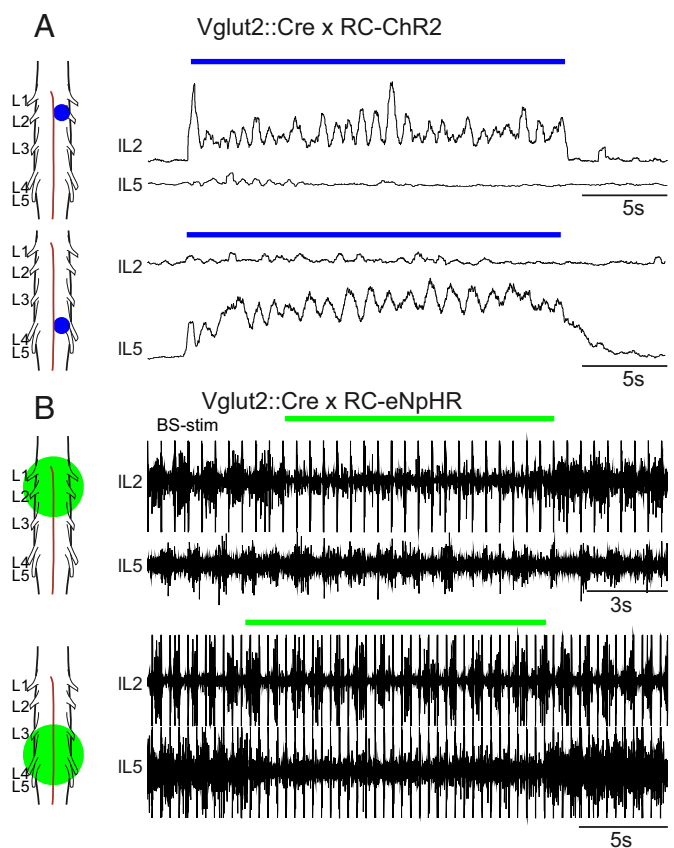
Fig. 4. Flexor and extensor networks can be independently bursting. (*A*) Activating glutamatergic neurons in a restricted area at the rostral lumbar cord can evoke locomotor-like activity exclusively in the flexor network (*Upper*), whereas similar activation at more caudal levels evokes locomotor-like activity exclusively in the extensor network (*Lower*). (*B*) Inhibiting glutamatergic neurons in the rostral lumbar spinal cord during brainstem evoked locomotor-like activity can turn off the flexor output from the L2 root while the L5 is still rhythmically active (*Upper*), albeit lower in frequency. Similar inhibition of glutamatergic neurons in the caudal lumbar spinal cord abolishes extensor output from the L5 root and causes a slight reduction in the frequency of the L2 locomotor bursts (*Lower*).

and extensor bursting exist independently and (*ii*) if so, can flexor- or extensor-related motor neuron pools burst independently of other flexor- or extensor-related motor neuron pools? To answer the first question, we used the *Vglut2::Cre; RC-ChR2* mice and shined small laser spots onto different parts of the cord. When a small spot (320 μm² in diameter) was placed at the L2 segment, it was possible to evoke rhythmic flexor bursting in the L2 ventral root without bursting in extensor-related L5 ventral root ($n = 9$, mean frequency = 0.93 Hz; Fig. 4A, *Upper*). Similarly, it was possible to evoke extensor bursting in L5 without bursting in L2 ($n = 7$, mean frequency = 0.94 Hz; Fig. 4A, *Lower*). Furthermore, it was possible to completely suppress either flexor or extensor bursting during brainstem-evoked locomotor-like activity in *Vglut2::Cre; RC-eNpHR* mice by inhibiting glutamatergic cells locally (Fig. 4B). Exposing the rostral lumbar cord to light (through a 10× or 50× objective) abolished L2 bursting, but L5 bursting continued; however, the frequency was reduced by 34% (0.13 Hz, $n = 4$, $P = 0.0012$; Fig. 4B, *Upper*). When the caudal lumbar cord was exposed to light in a similar manner, bursting in L5 was blocked and L2 bursting frequency was reduced by 17% (0.9 Hz, $n = 2$, $P = 0.0194$; Fig. 4B, *Lower*). Together, these experiments demonstrate that flexor and extensor bursting can exist independently, without the need for the reciprocity suggested by the half-center and asymmetric flexor-burst models.

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To answer the second question, we took advantage of the fact that the L4 ventral root projects to both flexor and extensor related muscles (40). A flexor-related burst (in phase with L2) and an extensor-related burst (in phase with L5) is seen as a double bursting pattern in the L4 ventral root when light is shined over the entire lumbar cord in *Vglut2::Cre; RC-ChR2* mice (Fig. 5A). With this stimulus paradigm, flexor-related bursting could be individually induced in the L2 or the L4 root. An example is shown in Fig. 5 B where L2 bursting was first evoked without bursting in L4 and L5 (*n* = 2, mean frequency = 0.98 Hz; Fig. 5 B, 1), then flexor bursting was evoked in L4 with extensor bursting in L5 demonstrated by their out-of-phase bursting (Fig. 5 B, 2; circular plot and time/frequency plot), and then finally extensor bursting in L4 in synchrony with extensor-related L5 bursting (Fig. 5 B, 3; circular plot and time/frequency plot). Moreover, it was possible to evoke bursting in L4 without bursting in L2 or L5 (Fig. S2). In this case, it is impossible to know whether L4 bursting is flexor- or extensor-related, but it clearly indicates that it is not necessary to have bursting in all flexors or all extensors at the same time.

The definitive confirmation of independent driven rhythmicity of motor neuron pools would be to get independent rhythmicity in two closely related muscles that fire in the same phase and reside close to each other in the spinal cord. We therefore recorded the activity in the nerves of a pure flexor related muscle (tibialis anterior) and a muscle that shows both flexor and extensor activity (vastus lateralis) (41, 42). The nerves to either the
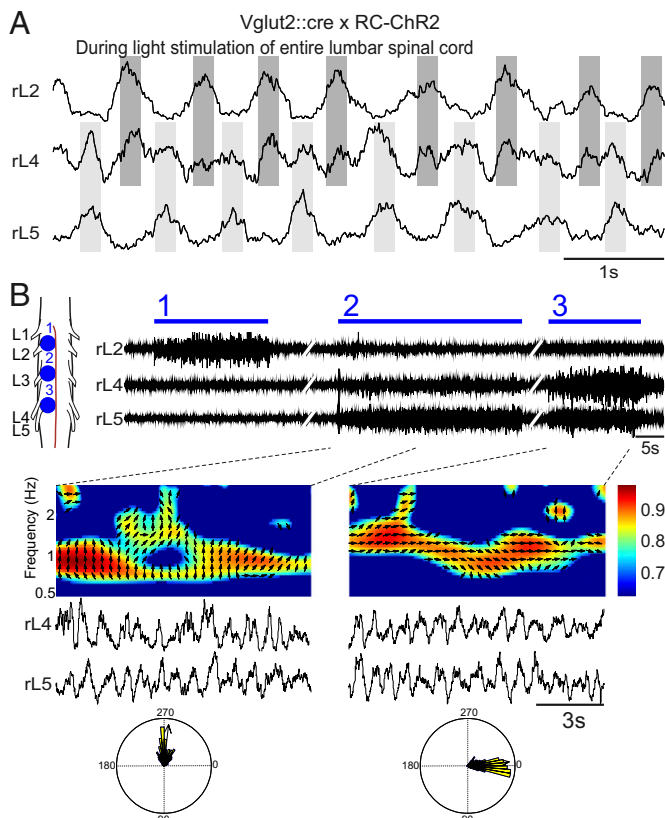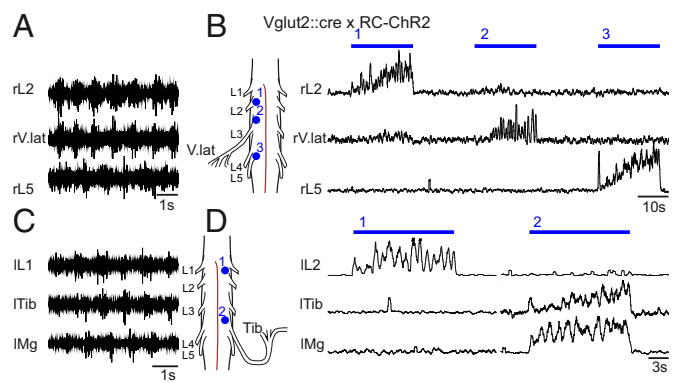
Fig. 6. Tibialis anterior or vastus lateralis nerve output can be independently rhythmic without activity in L2. Light simulation of the whole lumbar cord in the *Vglut2::Cre; RC-ChR2* mice elicits flexor-related activity in the nerve that projects to vastus lateralis (*A*) and tibialis anterior (*C*) muscles. Small spot stimulation shows that these motor neuron pools can be rhythmically active in flexor phase, independent from the rhythmic flexor activity in the L1 and L2 roots (*B* and *D*).

tibialis anterior or the vastus lateralis were dissected, and the nerve endings were cut right next to their respective muscle. Suction electrode recordings of these nerves during light stimulation of the entire lumbar cord showed them in phase with the L2-related flexor activity (Fig. 6 A and C). Small spot stimulation was able to elicit flexor activity in L2 separate from the flexor activity in vastus lateralis (coming from L3 ventral root, *n* = 2), or the tibialis anterior (coming from the L4 ventral root, *n* = 5) (Fig. 6 B and D). These experiments demonstrate that flexor-related motor neuron pools can burst independently of other close by flexor-related motor neuron pools.

Discussion

We have shown before (32) that activation of excitatory interneurons in the lumbar spinal cord is sufficient to evoke hindlimb locomotor-like activity. The present study demonstrates that activation of glutamatergic neurons is both sufficient and necessary for generating locomotor-like activity. In contrast, selective light-induced activation of inhibitory neurons will not elicit locomotion, but can instantaneously abolish any ongoing activity. Using this genetically driven functional dichotomy, we demonstrate that the mammalian hindlimb locomotor network can be segregated into multiple intrinsically rhythmogenic modules, each module composed of a presynaptic network driving a motor neuron pool, not unlike the unit burst model. Our experiments provide mechanistic insights into the functional organization of spinal rhythm-generating networks in the rodent and differentiate among several proposed models for rhythm-generation in the vertebrates.

Light-Induced Locomotor-Like Activity Is an Important Model for Locomotion. We have used intersectional mouse genetics to robustly express ChR2 and eNpHR in glutamatergic or GABAergic/glycinergic neurons combined with light stimulation of both global and restricted areas of the isolated spinal cord, using nerve activity as a proxy for locomotor-like activity. The cell-targeted, instantaneous, and spatially specific stimulation allows for a selective probing of the macroscopic architecture of the hindlimb locomotor network without having to induce lesions or use other irreversible manipulations. Using the optogenetic approach, we firmly establish that excitatory neurons are both sufficient and necessary for rhythm-generation in the rodent spinal cord, and that this function cannot be replaced by light activation of inhibitory neurons. Even with the excitatory network still present, activation of inhibitory neurons is unable to access this network to generate locomotor activity. When large areas of the cord are illuminated, the light-induced locomotor-like activity in the

Fig. 5. The mixed flexor and extensor motor output of L4 can be segregated by site-specific focal light stimulation. The L4 root carries a similar amount of motor output to extensor muscles as to the flexor muscles evident as a double burst pattern seen during locomotor-like activity (by whole preparation *Vglut2::Cre x RC-ChR2* stimulation in *A*). The flexor activity in L2 (*B*, *1*) and L4 (*B*, *2*) can be independently activated depending on the site of focal light stimulation. The L4 output in *B2* is flexor-related because it has a phase lag to L5 output (left circle plot). The extensor activity in L4 is also demonstrated by yet another stimulation spot (*B*, *3* and right circle plot).

Vglut2::Cre; RC-ChR2 mice is robust, with all of the characteristics observed during drug-induced or neural-evoked locomotor-like activity, including the mixed flexor-extensor output in the L4 root (39, 43, 44). Moreover, light-induced locomotor-like activity has a higher burst frequency than seen with other methods (up to 2.7 Hz), is well tuned, and is instantaneously turned on with the appropriate pattern.

Drug-induced locomotor-like activity is readily evoked and can be maintained for hours, permitting long timespan analysis in wild type as well as mutant and lesioned animals (45–50). Although the activity most likely uses the same networks as under in vivo conditions, an obvious drawback is that the drugs act upon every neuron in the spinal cord, not all of which are necessarily components of the locomotor network. Focal optogenetic stimulation of the spinal cord may have a potential to bypass these problems.

A Network with Multiple Rhythmogenic Flexor and Extensor Modules Is the Best Description of the Rodent Hindlimb CPG. Having established the robustness of optogenetic methods for activation or inactivation of the network, we could directly use it to address the macroscopic architecture of the network. Our study directly refutes several proposed models and favors one in particular.

The classical half-center model posits that the activity in both flexor- and extensor-networks are mutually dependent and both essential for rhythm generation. In this model, the individual half-centers are unable to burst without reciprocal activity. This organization clearly contrasts with our present findings that the flexor and extensor networks on the same side of the cord can be rhythmically active independently. Furthermore, multiple independent flexor and extensor rhythm generators can be activated in isolation. One could envision that the half-center would be structured over the left and right side of the cord instead. Such a structure would be consistent with the drop in locomotion frequency seen when a cord is bisected into two hemicords (38, 39), and with a bilaterally shared network proposed in turtles (51). The frequency change would then depend on a loss of commissural input. A recent study also found that the swimming CPG in the *Xenopus* tadpole embryo (stage 37/38) relies on bilaterally shared half-center organization for rhythm generation (52). However, our results clearly show that in the rodent CPG, similar to the lamprey (53), locomotor-like bursting can be induced unilaterally without any activity on the contralateral side or after the ventral commissure was cut and, thus, precluding any crossing signals.

The asymmetric flexor-burst model relies on a single rhythmogenic structure that is active during flexor bursts and, at the same time, inhibits tonic activity in extensors. In our results, we find support for the flexor rhythm being both faster and more readily evoked than the extensor rhythm. However, we also find that we can evoke bursting in extensors without any activity in the flexors, concluding that the hierarchical relationship between the two networks is not the characteristic that generates rhythmicity in the extensors but rather allows the endogenous rhythm to proceed in a coordinated fashion.

A modular network with a distributed burst capability is the organization that best accounts for the ability to evoke rhythmic activity in a limited population of the hindlimb motor neuron pools, as observed in the present study. In contrast to modules of synergists or muscles surrounding a single joint, we describe a module as a presynaptic network that appear to be able to drive individual motor neuron pools in isolation. This description is not unlike the unit burst generator model, first hypothesized for the cat locomotor network (18). The present experiments provide a direct demonstration of this model.

The best test for the existence of a modular network is to independently evoke rhythmicity in two closely related modules and observe the locomotor-like activity in the two corresponding motor neurons pools. The tibialis anterior and vastus lateralis motor neuron pools can fire in the flexion phase during normal locomotion and could be successfully separated from the flexor-related bursting in the L2 ventral root. This observation is

striking evidence for separate rhythmic modules given that the vastus lateralis motor neuron pool resides in the L3 segment, close to the *Iliopsoas* motor neuron pool that is recorded from the L2 ventral root.

Our previous experiments have indicated that an inhibitory premotor network organized in a half-center–like fashion can coordinate and produce combined flexor and extensor motor output in response to drugs (50). Moreover, several half-center motifs have been characterized by intracellular recordings during locomotion (54). These elements are probably essential for patterning the rhythmic output and are not disputed by the results in this study. However, this connectivity does not preclude that the rhythmicity itself is locally generated. Intrinsically rhythmic modules may also lead to a more flexible system that allows for different rhythmic patterns of activity, for instance scratching or backward walking (55). We therefore conclude that the most basic units of the locomotor CPG in rodents are independent rhythmogenic modules that are interconnected during locomotion to ensure a coordinated output.

Methods

All experiments were approved by the local ethical committee (Stockholm's Norra Forsöksdjursnämnd) and performed in accordance with European guidelines for the care and use of laboratory animals.

Mice. The transgenic lines described were as follows: *Vglut2::Cre* (34), *Rosa26-CAG-LSL-eNpHR3.0-EYFP-WPRE*, and *Rosa26-CAG-LSL-ChR2-EYFP-WPRE* (33). The *VIAAT::Cre* mouse is constructed by using BAC-technology; the production details are published in *SI Methods*.

In Vitro Experiments. Mice aged 0–4 d were used in all experiments. The pups were anesthetized in Isoflurane, decapitated, and eviscerated before the spinal cord with or without the brainstem attached was isolated in ice-cold low calcium Ringers solution [oxygenated 95% (vol/vol) O_2/5% (vol/vol) CO_2] that contained 111 mM NaCl, 3 mM KCl, 11 mM glucose, 25 mM $NaHCO_3$, 3.7 mM $MgSO_4$, 1.1 mM KH_2PO_4, and 0.25 mM $CaCl_2$ at pH 7.4. In most experiments, the lumbar ventral roots were cut at the exit point from the vertebrate canal. In some experiments, peripheral nerves to the vastus lateralis and tibialis anterior muscles were dissected out before the spinal cord was isolated with the peripheral nerves still attached. The dorsal roots were cut in all cases at the exit point of the vertebrate canal. The isolated spinal cord with nerves attached was transferred to a recording chamber that was continuously perfused with normal Ringers solution and contained 110 mM NaCl, 3 mM KCl, 11 mM glucose, 25 mM $NaHCO_3$, 1.25 mM $MgSO_4$, 1.1 mM KH_2PO_4, and 2.5 mM $CaCl_2$ oxygenated 95% O_2/5% CO_2 to obtain a pH of 7.4. All recordings were done at room temperature.

Drugs. In some experiments, NMDA (7 μM; Sigma) was used in combination with serotonin (5-HT, 8 μM, Sigma) to induce locomotor-like activity in vitro. CNQX (10 μM; Sigma) and AP5 (30 μM; Sigma) were used together to block glutamatergic neurotransmission during ChR2 intracellular recordings.

Neural-Evoked Locomotor Activity. The protocol for stimulating descending fibers inducing locomotor-like activity was similar to the one used in previous studies (56).

Recording of Locomotor Activity and Analysis. The locomotor activity was recorded with suction electrodes attached to the L2 and the L5 lumbar roots on either side of the cord or to the cut end of peripheral nerves. The nerve activity was band-passed filtered at 100 Hz to 1 kHz and sampled by using Axoscope 10 (Molecular Devices) at 1 kHz. Time/frequency plots were generated by using the Spinalcore software (57). The software uses morlet wavelet algorithms on rectified data to derive the frequency component of the time series data recorded from each root. The time/frequency data are plotted only when the two signals are significantly coherent. The phase relationship between the signals is then presented as a vector field. The color coding shows the coherence between the two signals.

Intracellular Recordings. Whole-cell recordings ware performed in acute 300-μm slices. Patch electrodes had resistances of 5–8MΩ. Electrodes contained 128 mM K gluconate, 10 mM Hepes, 0.0001 mM $CaCl_2$, 1 mM glucose, 4 mM NaCl, 5 mM ATP, and 0.3 mM GTP at pH 7.4. Whole-cell recordings were performed at room temperature by using a Multiclamp 700B amplifier

(Molecular Devices) and acquired by using pClamp software (Clampex v.10, Molecular Devices).

Light Stimulation of Cord. Light stimulation was executed through a 5×, 10×, or 50× microscope objective through a 450- to 490-nm band-pass filter for ChR2 or a 536- to 556-nm band-pass filter for eNpHR. For more restricted stimulations of ChR2-positive cells, we used a 473-nm laser scanning system (UGA-40; Rapp Optoelectronic) to illuminate spots ranging from 320–1,000 μm²

with an intensity ranging from 0.07–1.5 mW/mm² as measured with a laser power meter (Coherent).

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