Biochemical and Biophysical Research Communications 396 (2010) 11–18

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Probing spinal circuits controlling walking in mammals

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ARTICLE INFO

Article history:
Received 11 February 2010

Keywords:
Central pattern generator
Hb9
Chx10
GATA 3
En1
Sim1
Optogenetic
Interneuron
Transgenic mice

ABSTRACT

Locomotion in mammals is a complex motor act that involves the activation of a large number of muscles in a well-coordinated pattern. Understanding the network organization of the intrinsic spinal networks that control the locomotion, the central pattern generators, has been a challenge to neuroscientists. However, experiments using the isolated rodent spinal cord and combining electrophysiology and molecular genetics to dissect the locomotor network have started to shed new light on the network structure. In the present review, we will discuss findings that have revealed the role of designated populations of neurons for the key network functions including coordinating muscle activity and generating rhythmic activity. These findings are summarized in proposed organizational principles for the mammalian segmental CPG.

1. Introduction

Motor behaviors, like all brain functions, are produced by activity in dedicated neuronal networks. For locomotor behaviors in vertebrates, most of the aspects of the actual timing and coordination of the rhythmic muscle activity are generated by neuronal circuits in the spinal cord, often referred to as central pattern generators or CPGs. Studies in lamprey and *Xenopus* tadpole have been able to reveal the organization of swimming circuits in great detail using classical electrophysiology [1,2]. Analysis of these CPGs has shown that the core of the network consists of groups of excitatory and inhibitory neurons that serve designated roles in the network operation. Understanding the operation of CPGs controlling locomotion in mammals has been a significant challenge to neuroscientists, partly due to the large number of cells in the mammalian spinal cord as compared to the lamprey and tadpole spinal cord. However, recently, considerable advance has been gained in understanding the organization of the mammalian walking CPG. This progress has, to a large degree, been fueled by experiments performed in the isolated spinal cord preparation from neonatal rodents [3–8]. This preparation produces excellent conditions for electrophysiological network analysis, similar to what has been done for the vertebrate swimming CPGs. Moreover, interneurons in the cord can be divided into major subgroups based on their dynamic expression of transcription factors [9,10]. The molecular characterization of these early neuronal cell populations has provided a new basis for dissecting the neuronal circuits that control locomotion in the mammalian spinal cord. In this short review, we will discuss these experiments that have used electrophysiology and molecular genetics in the rodent to define the functional role of designated classes of neurons in the mammalian CPG. These findings will be summarized in a proposed organizational and molecularly delineated diagram of the segmental mammalian CPG.

2. Basic network organization

Limbed locomotion in mammals involves recurring activation of flexor and extensor muscles within a limb, and coordinated activity between the left and right legs. The isolated spinal cords from newborn rodents can produce the basic aspects of the locomotor behavior *in vitro* when appropriately stimulated with neuroactive drugs (Fig. 1) that mimic the descending inputs to the CPG from the brainstem or when initiating neural pathways are stimulated. The key features of the rodent locomotor network are the: (1) rhythm-generation, (2) flexor–extensor alternation, and (3) left–right coordination [5]. Below we will consider these network components in separate sections before bringing the findings together in a final synthesis of the network structure. This description will focus on the lumbar spinal cord that controls hindlimb movements in rodents.

3. From left to right

The neurons that are directly involved in coordinating left–right motor activity during locomotion are commissural interneurons

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doi:10.1016/j.bbrc.2010.02.107

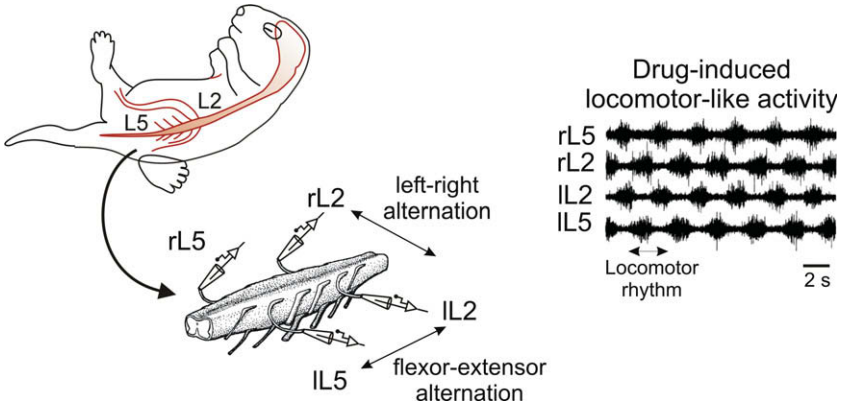

Fig. 1. Schematic of rodent *in vitro* spinal cord preparation. Hindlimb locomotor-like activity is seen as alternation between flexor-related bursts in lumbar (L) segment 2 and extensor-related bursts in L5 on the same side of the cord and as intrasegmental left–right alternation (lL2/rL2 and lL5/rL5) (adapted from [6]). The rhythm is determined as burst onset to burst onset.

(CINs) characterized by having axons crossing the midline. Commissural interneurons located within the CPG region in the rodent lumbar spinal cord have distinct axonal projection patterns that are *inter*segmental (ascending, descending, or both ascending and descending) or *intra*segmental [11]. The well-defined projection patterns make commissural interneurons a prime target for detailed electrophysiological connectivity studies and have provided a vantage point for network studies in the rodents.

The first group of commissural interneurons targeted in the rodent spinal cord was the descending commissural interneurons located in the rostral lumbar segments and projecting to the lower lumbar spinal cord [12,13]. Analysis of firing patterns and synaptic inputs from these descending commissural interneurons to caudally-located motor neurons revealed a network structure that was not supporting left–right alternation. Rather, rostrally-located descending commissural interneurons serve a role in binding flexor and extensor motor neurons diagonally across the cord into synergies. To serve this role, the activity of subgroups of descending commissural interneurons is orchestrated precisely to provide direct excitation, direct inhibition, or indirect inhibition during the appropriate locomotor phase. Connectivity studies suggest that similar coordinating systems may be active during cat locomotion [14–17].

Studies of the *intra*segmental pathways in rodents have revealed a dual inhibitory system that may be directly involved in left–right alternation [18] (Fig. 2A). One pathway in this dual inhibitory system is mediated by glycinergic/GABAergic commissural interneurons (CINi) that project onto contralateral motor neurons while the other pathway is mediated by glutamatergic commissural interneurons (CINei) which provide indirect inhibition of contralateral motor neurons (MN) via inhibitory interneurons including Renshaw Cells (RC) and unknown inhibitory neurons [18,19]. Glutamatergic commissural interneurons (CINe) also project directly to contralateral motor neurons [18]. This single direct excitatory pathway might be active during conditions of synchronous activity. Both the dual inhibitory and single excitatory pathways are supposed to connect to CPG neurons on the contralateral side (indicated with dotted lines). Recent anatomical studies using a number of transgenic mice have confirmed the triple transmitter phenotype of the commissural interneuron system [20].

Genetic manipulation of specific classes of commissural interneurons has provided further evidence for the proposed organization of the intrasegmental left–right coordination networks. In these experiments, the early molecular determinations of spinal interneurons are used to genetically silence, ablate or block synaptic connectivity in groups of neurons. Differentiation of spinal neurons is orchestrated by morphogens secreted from the ventral floor plate and dorsal roof plate [9]. The temporal and spatial concentration gradients of these morphogens in the ventro-dorsal axis of the developing cord define an early neuronal expression of transcription factors. Based on this expression pattern, four major subclasses of interneurons, called V0, V1, V2, and V3 interneurons, have been defined in the ventral spinal cord [9,10,21] in the area where the locomotor CPG is localized [22]. Two of these molecularly defined populations, the V0 and the V3 interneurons, are mainly commissural interneurons. The V0 interneurons express Dbx1 (Developing brain homeobox 1) early in development and develop later into (Fig. 2B, left) glutamatergic Evx1 (Even-skipped homeobox 1)-positive ventral V0 ($V0_V$; about 25%) and glycinergic/GABAergic Evx1-negative dorsal V0 ($V0_D$; about 70%) commissural neurons [23–25] and a small population (about 5%) of ipsilaterally-projecting glutamatergic ($V0_C$) and acetylcholinergic ($V0_C$) Pitx2 (Pituitary homeobox 2)-positive neurons [26]. V3 interneurons express Sim1 (Single-minded homolog) and are all excitatory and predominantly contralaterally projecting (85%) [27] (Fig. 2B, left).

In mice where the V0 population is reduced in number by knocking out the fate-determining transcription factor Dbx1, the drug-induced locomotor-like activity in the isolated spinal cord shows a disruption in normal left–right alternation with periods of synchrony intermingled with normal alternation [25]. Although there was not a full penetrance of the phenotype, this study suggested that commissural V0 interneurons are involved in segmental left–right alternation during locomotion. In support of this hypothesis, it was found that when the majority of V0 interneurons was ablated early in development by selectively driving the expression of diphtheria toxin A (DTA), the resulting offspring had a hopping rabbit-like gait [28]. How do these findings fit with the proposed scheme for left–right alternation? The combined V0 commissural interneuron population is composed of a mixed population of glutamatergic ($Evx1^+$, $V0_V$; 25%) and GABAergic/glycinergic ($Evx1^-$, $V0_D$; 70%) [25] neurons and therefore has the necessary transmitter-phenotypes needed for the dual inhibitory segmental CIN system described above (Fig. 2A). Moreover, inhibitory V0 commissural interneurons have been shown to project directly to motor neurons [25]. We therefore propose that a major component of the dual inhibitory crossed pathways is composed of the excitatory (indirect pathway $V0_V$, Fig. 2B) and inhibitory (direct pathway; $V0_D$ Fig. 2B) V0 commissural interneurons. In the absence of the V0 commissural interneuron systems the gait becomes locked into hopping, possibly by leaving the excitatory commis-

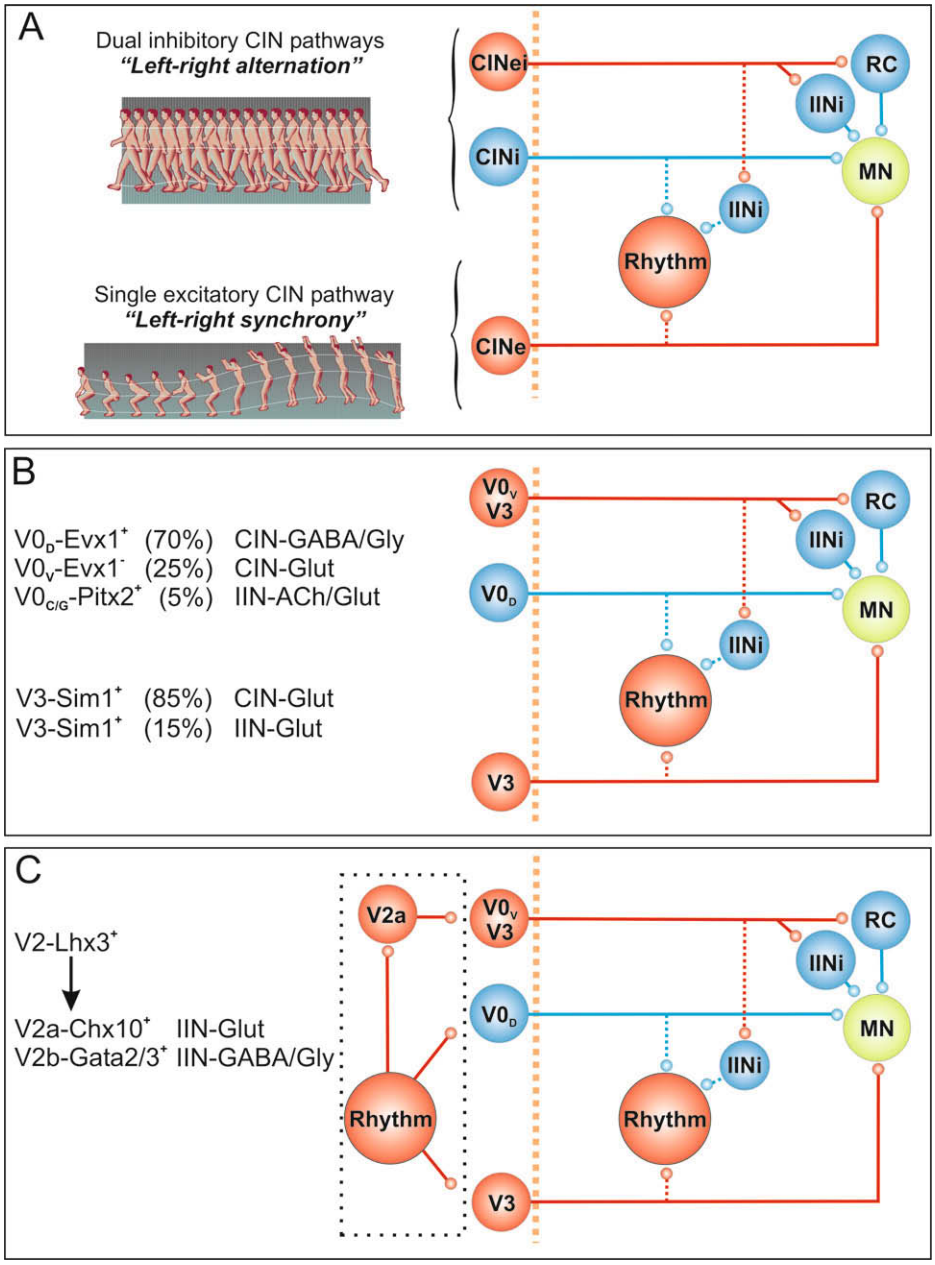

Fig. 2. Intrasegmental left–right coordinating circuits in the rodent CPG as determined by electrophysiology and molecular genetics. (A) A dual inhibitory system involved in left–right alternation is acting directly via inhibitory CINs (CINi), or indirectly, via excitatory CINs (CINei) on contralateral motor neurons (MN). A single excitatory system (CINe) involved in synchronous activity is acting directly on motor neurons. To obtain left–right coordination during locomotion, these crossed connections should also connect to the rhythm-generating core on the other side of the cord as well as the corresponding commissural interneuron (these connections have not yet been determined experimentally and are indicated with dotted lines). Inhibitory neurons are blue. Glutamatergic neurons are red. Dotted line indicates the midline. IINi: Ipsilaterally-projecting inhibitory neuron. Modified from Ref. [18]. (B) Defined molecularly-derived CINs belonging to ventral classes drive the different legs of the dual inhibitory CIN pathway and single excitatory CIN pathway. Left, Dbx1 positive progenitor cells generate glutamatergic (Glut) Evx1$^+$ CINs (25%), called $V0_V$, GABAergic/Glycinergic (GABA/Gly) Evx1$^-$ CINs (70%), called $V0_V$, and a small population of ipsilaterally-projecting neurons ($V0_C$/$V0_G$, see text). Sim1$^+$ positive V3 neurons are glutamatergic and, for the most part, CINs (85%). Right, Proposed scheme for the V0- and V3-related CINs in the left–right coordinating network based on neuronal ablation/silencing and gene-silencing experiments as well as genetic tracing studies (e.g., [25,27,28]) (see text for details). (C) The drive of left–right coordinating pathways is mediated through multiple ipsilaterally-projecting glutamatergic neurons including the V2a population. Left, Lhx3 positive progenitor cells generate ipsilaterally-projecting neurons that are either glutamatergic and called V2a interneurons or GABAergic/glycinergic and called V2b interneurons. Right, Proposed scheme for the drive (within the rectangle) of the dual inhibitory and single excitatory pathways based on genetic ablation experiments, genetic tracing studies and electrophysiological studies [33,34,36,37]. Note that the V2a neurons are dispensable for rhythm-generation. The model encompasses speed related changes in coordination (see text for details) (adapted from Ref. [33]).

sural interneuron pathways responsible for segmental left–right synchrony intact.

The role of V3 neurons in left–right coordination was tested in experiments where synaptic release from Sim1$^+$ neurons was permanently blocked or the neuronal activity was acutely reduced by genetically driven expression systems [27]. Both procedures effectively removed or reduced the influence of the V3 neurons during network activity. Under these experimental conditions, locomotor activity was disrupted with larger variability in the locomotor burst amplitude and period, and imbalance of motor activity on the left and right side of the cord. The Sim1$^+$ neurons were shown to have anatomical connections to the Renshaw Cells, inhibitory Ia

interneurons and motor neurons. Taken together these findings suggest that the V3 commissural interneurons play a role in coordinating precision and regularity of motor neuron activity across the cord although their role in left–right alternation is minor compared to the V0 commissural interneurons. However, the projection to Renshaw Cells and Ia interneurons may suggest that a subpopulation of $Sim1^+$ neurons, together with the $Evx1^+$ neurons, makes up the indirect pathway in the dual inhibitory commissural pathway (Fig. 2B). Deleting one of these neuronal populations from this pathway would therefore not be enough to upset the left–right alternation. The direct projection from $Sim1^+$ neurons to contralateral motor neurons indicates that the $Sim1^+$ commissural interneuron pathways are active under conditions of left–right synchrony (hopping; Fig. 2B). The study by Zhang et al. [27] did not test this hypothesis directly by determining whether or not left–right synchrony was disrupted after blocking all inhibitory pathways. However, mice with midline axon guidance defects that spare V3 CIN crossings show a hopping phenotype similar to the one observed in Dbx1-DTA mice, suggesting that the V3 commissural interneurons indeed are responsible for left–right synchrony [29]. Thus, the electrophysiological and genetic connectivity studies of commissural interneurons and the genetic loss of function studies of the V0 and V3 populations converge towards a common network structure controlling left–right coordination.

Immediate questions that arise from these studies are: (i) Which neurons drive these commissural pathways? (ii) Are the dual-inhibitory pathways driven by one set of neurons? and (iii) Are the CINs being driven directly by the rhythm-generating neurons? Some answers to these questions come from recent experiments that have ablated the spinal V2a interneurons. V2a interneurons are derived from progenitor neurons that express Lhx3 (LIM/homeobox 3) early in development and then differentiate into V2a and V2b classes, expressing the transcription factors Chx10 (Ceh-10 homeodomain-containing homolog) and Gata2/3 (GATA binding protein), respectively [9,30] (Fig. 2C, left). The V2a interneurons are strictly glutamatergic and project ipsilaterally, while V2b interneurons are inhibitory and ipsilaterally projecting [31,32] (Fig. 2C, left). When Chx10 neurons were selectively ablated by the expression of DTA under the control of the Chx10-promotor, the locomotor frequency and motor burst amplitude of drug-induced locomotor-like activity became more variable than it was with the V2a interneurons present [33]. Moreover, left–right alternation was disrupted in the Chx10-DTA mice, which was seen as a decoupling of the activity between the two sides of the cord [33]. These findings lead Crone et al. [33] to propose that the ipsilaterally-projecting excitatory Chx10 neurons drive both of the dual inhibitory CIN pathways involved in normal left–right alternation. However, a subsequent study showed that disruption of left–right alternation was most pronounced at moderate locomotor frequencies and that left–right alternation was normal at lower locomotor frequencies [34]. Together these findings suggest that the Chx10-mediated drive to the segmental left–right alternating system dominates at moderate frequencies while at lower frequencies other neurons provide the dominant drive to the segmental left–right alternating system. Crone et al. [33] showed that Chx10 neurons connect to $Evx1^+$ positive commissural interneurons. We therefore suggest that the Chx10 neurons only project to the indirect inhibitory pathway (the $Evx1^+$ pathway) and that other neurons (e.g., the rhythm-generating excitatory neurons) drive the direct inhibitory pathway [35] (Fig. 2C). The firing patterns of Chx10 neurons during locomotor-like activity are supportive of such a role [36,37]. These recordings also show that Chx10 neurons are active, even at lower locomotor speeds. This indicates that, although the indirect dual-inhibitory pathway driven by V2a interneurons might be dominant only at high speeds, it contributes to left–right alternation during all locomotor speeds [35].

The V2a ablation study also showed that the crossed pathway involved in segmental synchrony is functional [33,34], suggesting that this pathway is driven by excitatory neurons other than the V2a interneurons (Fig. 2C). It is also noteworthy that the rhythm-generation persisted in the absence of V2a interneurons, although there was an increased variability in the motor burst amplitude and locomotor frequency. These effects suggest that V2a interneurons are not the main source of rhythm-generation in the mammalian spinal cord, although spinal Chx10 neurons may provide direct excitatory inputs to motor neurons and/or have some weak permissive effect on the rhythm-generating core of the network [33] (see Fig. 3).

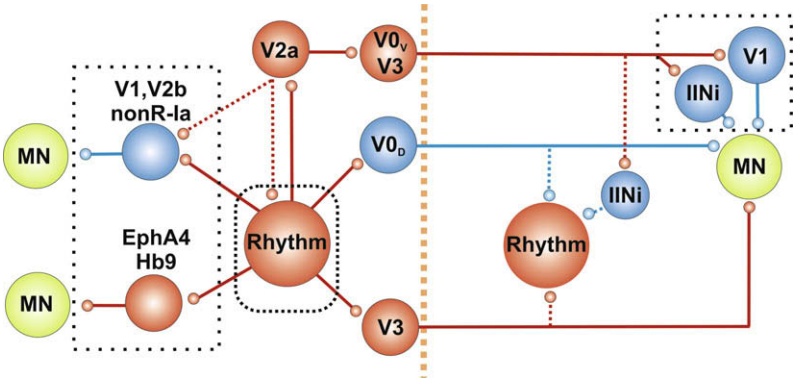

Fig. 3. Overall segmental organization of the rodent CPG including ipsilateral circuits activating motor neurons. Left–right circuits are described in Fig. 2. Motor neuron rhythmicity is generally driven by alternating excitation and inhibition. Rhythm-generating CPG interneurons (dotted circle) drive last-order inhibitory neurons. Electrophysiological and genetic studies have indicated that candidate inhibitory neurons are Ia interneurons, Renshaw Cells belonging to the V1 population, non-reciprocal Ia interneurons and possibly V2b neurons (dotted square ipsilateral side). Some inhibition is also mediated by crossed connections (dotted square contralateral side). V2a interneurons might also have connections to motor neurons and the rhythm-generating core on the ipsilateral side, although they are dispensable for both rhythm-generation and/or drive to motor neurons. Therefore, other types of ipsilateral excitatory neurons besides the Chx10 neurons have to generate the rhythm and drive motor neurons, directly or indirectly. Possible candidates for the last-order excitatory neurons are EphA4 and Hb9 positive neurons. The present summary diagram only shows connectivity at the segmental level and will only account for activity in one locomotor phase (e.g., L2 flexor motor neurons). During normal locomotion, flexors and extensors are activated in a reciprocal pattern. This presumably requires that the rhythm-generating core has both a flexor and an extensor center that is active in a reciprocal pattern. Such a feature is easily incorporated in the diagram by duplicating the ipsilaterally- and contralaterally-projecting neuronal populations. The complex timing of muscle activity within the limb is also not addressed in the diagram.

4. Flexor–extensor alternation and rhythmic inhibition of motor neurons

Execution of walking requires that flexor and extensor muscle activity is alternating within a limb. This alternating activity depends on inhibitory CPG networks, since flexors and extensors are activated in synchrony when all fast inhibitory transmission is blocked during locomotor-like activity [38]. These networks are, for the most part, ipsilateral because flexor–extensor alternation persists even in the hemicord (see [5] for references). Individual rodent motor neurons are also driven into rhythmicity by alternating synaptic glutamatergic excitation and glycinergic/GABAergic inhibition [39–41]. Some of this inhibition comes from the commissural interneurons but the main contribution is thought to be ipsilaterally generated (Fig. 3). Taken together these observations imply that ipsilateral inhibitory networks provide (i) rhythmic inhibition of motor neurons and (ii) reciprocal organization of flexor and extensor CPG networks on the ipsilateral side (see however [3]). These ipsilateral inhibitory network neurons are not well defined. Below we will consider these network components separately.

4.1. Rhythmic inhibition of motor neurons

Renshaw Cells and inhibitory Ia interneurons have long been proposed to be involved in motor neuron inhibition during locomotion because of their known projection to motor neurons and rhythmic firing during locomotion in mammals [19,42,43]. These neurons are thought to be derived from the V1 class of neurons that express En1 (Engrailed 1) [44]. The V1 class of neurons is purely glycinergic/GABAergic and ipsilaterally projecting. Renshaw Cells and Ia interneurons are thought to make up about 30% of the V1 neurons. However, rhythmic inhibition is still present in motor neurons when the entire V1 population is genetically ablated or silenced [45a]. This would exclude Renshaw Cells and Ia inhibitory interneurons as the main sources of rhythmic inhibition. However, a recent study has indicated that the Ia inhibitory neurons may originate from a non-V1 derived progenitor domain, still leaving the Ia interneurons as possible contributors to the rhythmic inhibition [45b]. Another source of rhythmic last-order inhibition recently defined in the mouse spinal cord is a group of GABAergic neurons located in the deep dorsal horn that have most of the characteristics of the so-called non-reciprocal interneurons found in the cat spinal cord [46]. Together this would indicate that the rhythmic inhibition of motor neurons is generated by several groups of molecularly-defined neurons, including V1 derived neurons, non-reciprocal Ia interneurons and possibly other inhibitory neurons like the V2b neurons (Fig. 3).

4.2. Coordination of flexor–extensor alternation

The flexor–extensor alternation is also not affected when the V1 population is genetically ablated or silenced [45a]. Rather this perturbation leads to a consistent slowing of the locomotor frequency. This finding indicates that ipsilateral inhibitory networks belonging to the V1 population are involved in setting the locomotor frequency in a way that is not yet resolved (see Discussion in [5]). The circuitry coordinating flexor–extensor alternation therefore still needs to be determined. Candidate neurons include the Gata2/3 positive V2b inhibitory neurons but it is possible that this function is controlled by more than one of the cardinal groups of molecularly-defined neurons (e.g., V1 and V2b).

5. Rhythm-generation and rhythmic excitation of motor neurons

Pharmacological studies have suggested that spinal glutamatergic neurons are essential for rhythm-generation in the mammalian walking CPG, as is the case in the swimming CPG (see [1,2,47]). Our recent experiments have shown this in a more direct way by using a transgenic mouse line that expresses the optically active protein Channelrhodopsin-2 (ChR2) in vesicular glutamate transporter 2 (Vglut2) positive neurons [48] (Fig. 4). Vglut2 is found in all glutamatergic neurons in the CPG region of the mouse spinal cord [20,32,49]. Light-stimulation of the spinal cord directly in ChR2–Vglut2 mice initiates and maintains locomotor-like activity, suggesting that glutamatergic neurons in the spinal cord are indeed responsible for rhythm-generation and provide the necessary drive in the network for it to be operational. Since a locomotor-like rhythm can be produced in hemi-sectioned cords, the glutamatergic neurons are likely to be ipsilateral although crossed fibers may be involved in setting the 'tone' in the rhythm-generating network [50–52]. Moreover, ipsilaterally-projecting neurons participate in providing rhythmic excitation of motor neurons [39–41].

5.1. Rhythm-generation

What neuronal types generate the rhythm and provide rhythmic drive in the network? Although genetically controlled elimination of the classes of ventral neurons (V0–V2) causes specific changes in the pattern of activity, none have been shown to abolish the rhythm-generation. Moreover, the Hb9 (homeobox 9) positive neurons that are glutamatergic, ipsilaterally-projecting, possess rhythmogenic properties, and are interconnected [49,53–57] but do not seem to fire in the right phase of the locomotor cycle to be the prime source of rhythm-generation [58]. Collectively these findings suggest that other glutamatergic neurons than those belonging to the V0–V2 classes and the Hb9 neurons are responsible for rhythm-generation. An alternative possibility is that the rhythm-generating neurons span several classes of the known molecularly defined groups of neurons making it difficult to specifically eliminate them from the network.

5.2. Rhythmic excitation of motor neurons

There are two molecularly defined groups of glutamatergic, ipsilaterally-projecting neurons that may provide direct excitatory connections to ipsilateral motor neurons and are rhythmically active at the peak of the ipsilateral motor activity. These are the EphA4 positive neurons [59] and the Hb9 positive neurons [53]. A possible functional role of these neuronal classes is therefore to provide direct rhythmic excitatory drive to motor neurons during locomotion (Fig. 3). However, many of neurons in these two classes do not project directly to motor neurons [49,59].

6. Neural initiation of locomotion

The CPG activity is normally turned on by command signals in descending pathways. In mammals both glutamatergic reticulospinal neurons and descending serotonergic neurons located in the lower hindbrain have been implicated in sending the command signal to activate the spinal CPG [60]. Experiments using the Vglut2–ChR2 mice have shown that photo-stimulation of glutamatergic neurons in the lower hindbrain is sufficient to activate the spinal locomotor CPG in mice [48] perhaps ascribing a lesser role to descending serotonergic fibers in initiating locomotion. It will be of tremendous interest to find the sites of action of descending glutamatergic initiating pathways in the CPG. At the moment the only known targets of reticulospinal neurons in rodents are motor neurons [61].

By using a conditional approach, it will be possible to express ChR2 in specific populations of glutamatergic hindbrain neurons including Chx10 neurons descending from the reticular nucleus

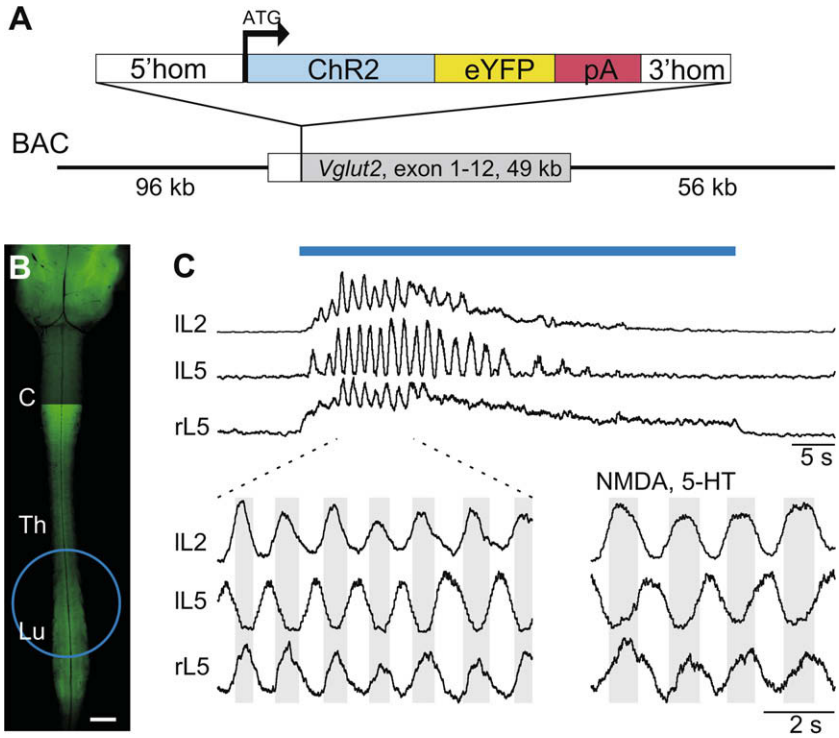

Fig. 4. Direct light-activation of excitatory Vglut2-expressing neurons in the spinal cord induces rhythmic locomotor-like activity. (A) Channelrhodopsin-2, a light-activated depolarizing channel isolated from algae, was fused to eYFP and recombined into a bacterial artificial chromosome vector containing the genomic region of the *vglut2* gene. Vglut2 is exclusively expressed in glutamatergic neurons in the CPG region in the lumbar spinal cord. (B) Distribution of YFP-positive cells in whole mount preparation of spinal cord (Lumbar, L; Thoracic, Th; Cervical, C), and brainstem (ventral side up, montage). (C) Continuous light-activation of the lower thoracic (Th12–13) and upper (L1–L2) lumbar spinal cord ((B) blue circle) generated locomotor-like activity ((C) top and second trace to the left) in the *in vitro* spinal cord preparation, seen as alternation between the left (l) and right (r) lumbar (L) ventral roots in the second (2) segment (*left–right alternation* (lL2/rL2)) and between the ventral roots in L2 and L5 segments (*flexor–extensor alternation* (lL2/lL5)). The light-induced activity was analogous to pharmacologically-induced rhythmic activity (5-HT in combination with NMDA) (C, lower right, same animal). Adapted from Ref. [48].

(see [34]). Since ablation of Chx10 neurons leads to the inability to initiate locomotion from hindbrain stimulation, it is very likely that Chx10 positive neurons in the hindbrain account for a strong component of the descending initiating system [34].

7. Neuromodulation

The motor activity can also undergo modulatory changes in frequency and amplitude. This neuromodulation includes monoaminergic [4,62,63] and GABAergic modulation [64,65] which is often extrinsic to the network. Using molecular genetics, a recent study has revealed the physiological role of modulatory neurons intrinsic to the spinal cord [26]. These neurons are the Pitx2^{+} and acetylcholinergic neurons, called $V0_C$, since they are derived from the V0 population. $V0_C$ neurons are shown to be the exclusive source of the well-known large acetylcholinergic C-boutons that were first discovered on motor neurons more than 40 years ago [66]. The $V0_C$ neurons are rhythmically active during locomotor-like activity and genetic inactivation of these cells reduces the amplitude of the locomotor output in a task-dependent way [26]. The $V0_C$ neurons therefore seem to represent a defined class of neuromodulatory neurons that are recruited when a behavioral setting requires stronger motor output.

8. Concluding remarks

A detailed picture of the network structure of the rodent CPG is beginning to emerge. Studies of the left–right coordinating systems have been efficient in probing the network function. Electrophysiological and molecular genetic studies of commissural interneurons have revealed a complex CPG network structure controlling left–right coordination, with designated neuronal pathways for left–right alternating and synchronous motor activity. Genetic ablation studies have defined the molecularly non-overlapping groups of ipsilaterally-projecting excitatory neurons involved in driving the left–right coordinating systems. The network structures generating rhythmic inhibition and excitation of motor neurons have not been defined in detail but a number of candidate neurons are known. The inhibitory circuitries coordinating reciprocal activity between flexors and extensors are yet to be defined. Similarly the excitatory interneurons directly responsible for generating the rhythm have so far escaped identification. The rhythm-generation capability seems to reside in glutamatergic neurons since genetically controlled activation of these neurons efficiently initiates and maintains the CPG activity. A major challenge for future studies is therefore to design experiments that can reveal the origin and the mechanisms for the rhythm-generation itself in the mammalian spinal cord, perhaps by taking into consideration that the rhythm-generation may be safeguarded by the interactions of several neuronal populations including ipsilaterally-projecting inhibitory interneurons and crossed pathways that can also set the tone of the locomotor speed.

Acknowledgments

The work in the Kiehn laboratory is supported by grants from the US National Institutes of Health (R01NS040795-09), the European Union (SPINAL CORD REPAIR), the Swedish Medical Research Council, Söderbergs Foundation, and Friends of Karolinska Institutet.

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