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Commentary

Deciphering the organization and modulation of spinal locomotor central pattern generators

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Summary

Networks within our spinal cord generate the basic pattern underlying walking. Over the past decade, much progress has been made in our understanding of their function in a variety of vertebrate species. A significant hurdle has been the identification of candidate populations of neurons that are involved in pattern generation in the spinal cord. Recently, systems neuroscientists in collaboration with molecular biologists have begun to dissect the circuitry underlying spinal locomotor networks. These advances have combined genetic and electrophysiological techniques using *in vitro* preparations of the mouse spinal cord. This review will discuss new advances in the field of spinal locomotor networks with emphasis on the mouse.

Many of the behaviors fundamental to animal life, such as breathing, chewing and locomotion, are rhythmic activities controlled by neuronal networks. Discerning which neurons are members of these networks, their synaptic connectivity and their individual electrophysiological properties is essential to our understanding of how rhythmic motor behaviors are produced. It is well known that the spinal cord contains the basic circuitry to produce locomotion. However, identifying neurons and connections within spinal networks is challenging because cells that comprise the locomotor network form part of a heterogeneous mix of interneurons within the ventral spinal cord. Recently, the merging of electrophysiological and genetic approaches has provided new tools to identify classes of interneurons within the spinal cord that contribute to network function. These new findings will be discussed in this review.

Key words: CPG, mouse, locomotion, spinal cord, monoamine, sensory.

Introduction to spinal locomotor networks and *in vitro* models

Many years ago, Graham-Brown found that coordinated flexor and extensor alternating movements could be produced in the absence of descending or afferent inputs to the lumbosacral spinal cord (Graham-Brown, 1911). This important finding has been confirmed in many species including humans, and the term ‘central pattern generator’ (CPG) has been coined to refer to the network of neurons that can produce patterned motor behavior in the absence of phasic inputs (Grillner, 1981). Now we know that many motor behaviors can be produced by spinal cord networks, including hopping, walking, swimming and scratching (Grillner, 1981). The patterns produced by spinal CPGs are remarkably sophisticated, and unique patterning of mono- and biarticular flexor and extensor muscles can be identified. An elegant example involves the use of decerebrate cats that were induced to step over a treadmill by electrical stimulation of an area surrounding the cuneiform nucleus (Grillner and Zangger, 1984). The stepping of the animal was compared in the presence and absence of phasic afferent input. Although the pattern became more variable without sensory input, the complex timing of flexor and extensor muscles during locomotor activity was maintained. Another example involves the use of cats that have been retrained to step with their hindlimbs following a full transection of their thoracic spinal cord (Barbeau and Rossignol, 1987). Overall, there were few differences in the stepping patterns produced by intact and spinally transected animals (Belanger et al., 1996). That said, it must be emphasized that CPGs are but one component of an integrated locomotor system (Rossignol et al., 2006).

The original half-centre model proposed by Graham-Brown consists of a flexor and an extensor half-centre coupled by mutually inhibitory interneurons. Oscillatory output from this half-centre is fed directly to pools of extensor and flexor motoneurons. However, during normal locomotion, leg muscles produce a diverse range of patterns that deviate from a simple flexor–extensor pattern. How spinal CPGs can

produce different patterns is a matter of some debate, but it is clear that the simple half-centre model cannot account for the observed diversity (Burke et al., 2001; Stein et al., 1998). One possibility is that multiple oscillators are flexibly coupled to produce different patterns (Grillner, 1981). Nevertheless, it is still difficult to explain double burst patterns within bifunctional muscles without an additional output layer being interposed between the oscillator and the motoneurons. Several investigators have proposed alternative models that have a patterning network that receives rhythmic drive from a separate layer of neurons in the spinal cord (Burke et al., 2001; Lafreniere-Roula and McCrea, 2005). The patterning network can, in principle, account for many of the complex patterns that are observed during stepping. To examine the validity of these models, we ultimately need to identify classes of interneurons that form the network responsible for the oscillatory drive of the CPG and to investigate the hypothesized downstream networks of premotor interneurons that generate the pattern. This is difficult but advances in genetic techniques along with current electrophysiological techniques are generating new optimism that the network underlying locomotion in mammals can be unraveled. Due to the large number of genetic models available, the mouse is emerging as an ideal model to study spinal network function. Since most of these studies currently use *in vitro* approaches, we will briefly discuss the types of preparations that have been tested and are used on a regular basis (Bonnot et al., 1998; Bonnot et al., 2002a; Jiang et al., 1999; Whelan et al., 2000).

Generally speaking, *in vitro* spinal tissue offers several advantages over *in vivo* approaches, including easy manipulation of the external bath medium, application of drugs that do not cross the blood–brain barrier, the use of calcium and voltage-sensitive optical recording techniques and the ability to reversibly manipulate the excitability of networks within discrete segments of the spinal cord (Bonnot et al., 2002b; Grillner, 2003; Roberts et al., 1998; Smith et al., 1988; Stein et al., 1998). There are at least three types of neonatal mouse spinal cord preparations that can be used to study locomotor networks. First, an *in vitro* preparation consisting of a thoracosacral spinal cord with attached hindlimbs can be dissected (Whelan et al., 2000). Since descending inputs are cut, the spinal CPG is usually activated using bath-applied drugs such as dopamine, NMDA and serotonin (5-HT). Monoamines, such as 5-HT, dopamine and noradrenaline, appear to provide a sustained high level of neuronal excitability necessary to recruit a sufficient number of neurons to activate the CPG (Christie and Whelan, 2005; Kiehn et al., 1999; Liu and Jordan, 2005; Madriaga et al., 2004) and may be important activators of spinal networks before other descending projections are fully developed (Branchereau et al., 2000). Following the addition of these drugs, one can see the hindlimbs flexing and extending out of phase with each other. These locomotor patterns can be recorded reliably by implanting wires into the muscle or by using suction electrodes to record neurograms from selected muscle nerves (Whelan et al., 2000) (Fig. 1B). An advantage of using this type of preparation is that the pattern of activity can be compared with data from *in vivo* studies (Fig. 1A). One can reduce the preparation even further by dissecting away all tissue except for the spinal cord and the dorsal and ventral roots (Jiang et al., 1999). By comparing the pattern produced in the ankle extensor and flexors with ventral root recordings, it was found that a signature of locomotor-like activity can be obtained by typically recording from the left and right lumbar 2 (L2) and 5 or 6 (L5/6) ventral roots (Whelan et al., 2000). Neurograms from the L1–3 segments show that bursts occur during the flexor phase, while bursts from L5–6 occur during the extensor phase. A caveat is that the evoked pattern does not provide the detail that can be obtained using the semi-intact preparation (compare the pattern in Fig. 1B with that in Fig. 1C) (see Cowley and Schmidt, 1994). Nonetheless, the excellent viability of the isolated *in vitro* spinal cord preparation has made it a convenient choice for many labs.

While the use of externally applied drugs to activate the CPG is effective, it has the drawback of indiscriminately activating cells in the network. These drugs also bind and activate extrasynaptic receptors not normally recruited *in vivo*. Another issue is that in developing spinal cord preparations, the receptors are often expressed in advance of fibers being present. Thus, it is not clear that the observed activation of networks could occur in developing animals. One approach to tackle this problem is to electrically stimulate afferents that activate spinal circuits (Whelan et al., 2000). This technique is useful since it relies on endogenous release of neurotransmitters and neuromodulators to excite spinal networks. Stimulation of the cauda equina or lumbar dorsal root afferents with trains of electrical pulses can effectively activate thoracosacral spinal CPGs (Fig. 2A), producing consistent locomotor-like patterns (Bonnot et al., 2002a; Whelan et al., 2000). Most likely due to synaptic fatigue, it is necessary to provide a rest period of a few minutes after each stimulus bout. Nevertheless, since the preparation lasts for many hours, this allows for multiple bouts of locomotor activity to be recorded. Our preliminary data indicate that cauda equina stimulation evokes a coordinated cervicolumbar pattern (Fig. 2B), suggesting that, early in development, propriospinal projections are sufficiently developed to drive cervical networks in the mouse.

In summary, locomotor networks can be reliably activated and modulated in the *in vitro* mouse spinal cord preparation using a variety of pharmaceutical or electrical stimulation techniques. In the remaining part of the review, we will examine how these preparations are being used together with molecular approaches to dissect the circuitry underlying CPGs.

Identifying the component parts of mammalian CPGs

One approach to examine whether a neuron contributes to network function is to alter activity within the cell and examine the output of the network. This method has been successfully employed in many invertebrate systems to map out network connectivity (Marder and Bucher, 2001). However, in vertebrate

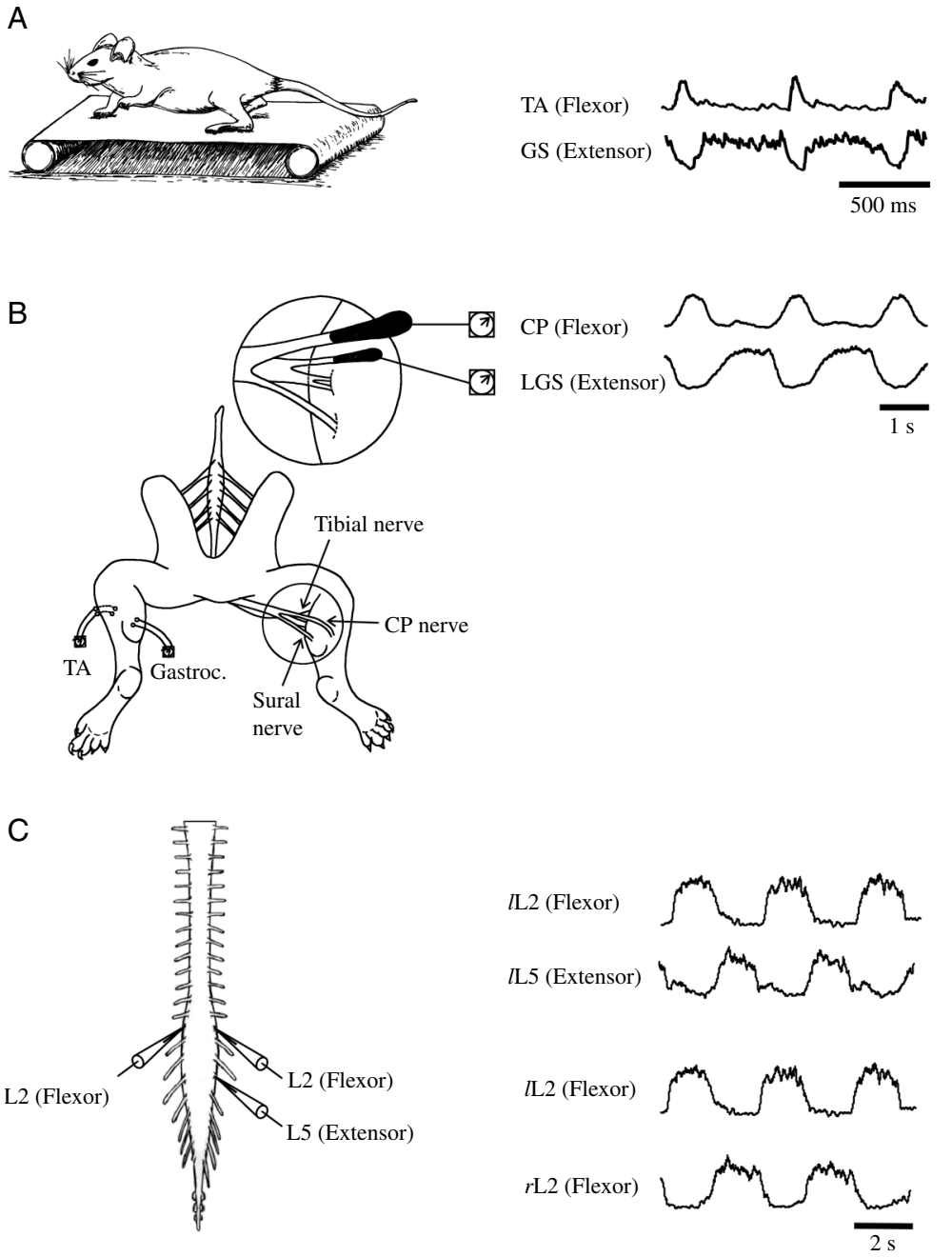

Fig. 1. (A) Pattern of flexor and extensor muscle activity recorded from a conscious adult mouse walking on a treadmill. Adapted from Pearson et al. (Pearson et al, 2005) with permission. (B) A hindlimbs-attached preparation can be used with electromyographs (EMGs) or electroneurograms (ENGs) to record activity from spinal networks (Pearson et al., 2003; Whelan et al., 2000). The thoracosacral spinal cord is preserved in this preparation. ENGs can also be recorded from the lateral gastrocnemius (LGS) and common peroneal (CP) nerve, which allow flexor and extensor activity to be recorded as shown in the example (Whelan et al., 2000). Note that the flexor and extensor pattern produced by the *in vitro* neonatal mouse preparation is qualitatively similar to that produced by adult mice walking on a treadmill. (C) Isolated spinal cord preparation commonly used to record locomotor-like patterns *in vitro*. Alternation between ipsilateral L2 and L5 ventral root recordings corresponds to flexor–extensor activity, while segmental alternation between the left (*l*) and right (*r*) L2 roots reflects left–right alternation (Whelan et al., 2000). Rhythm evoked using a combination of serotonin (5-HT), *N*-methyl-D/L-aspartate (NMA) and dopamine (see Jiang et al., 1999). TA, Tibialis anterior; GS, gastrocnemius

networks, interneuronal populations need to be identified and manipulated in order to perturb a spinal CPG. This is a serious obstacle to overcome in mammals, where large heterogeneous populations of interneurons contribute to network function. Recently, developmental neuroscientists have succeeded in generating mice where populations of candidate neurons contributing to spinal CPG function can be identified and manipulated (Goulding and Pfaff, 2005). During embryogenesis, five ventral progenitor cell domains are formed by a dorsoventral diffusion gradient of a protein known as sonic hedgehog (Shh) (Briscoe and Ericson, 2001; Lee and Pfaff, 2001). The distinct classes of progenitor cells give rise to motoneurons and four classes of ventrally located interneurons (V0–3). These cell populations ultimately form discrete classes of interneurons that form reflex or locomotor circuits. For example, V0 cells form commissural interneurons that project rostrally for 1–4 segments. On the other hand, V1-derived cells project ipsilaterally for short distances, usually within a segment, and become inhibitory neurons, such as Renshaw cells, and likely Ia inhibitory interneurons (Sapir et al., 2004). New evidence suggests that V1 interneurons control the speed of locomotion (Gosgnach et al., 2006). V3 cells give rise to commissural excitatory cells (Narayan et al., 2004) that synapse in the lumbar cord onto contralateral motoneurons. Finally, V2 cells project ipsilaterally for longer distances (1–4 segments) (Fig. 3) and may control flexor/extensor alternation.

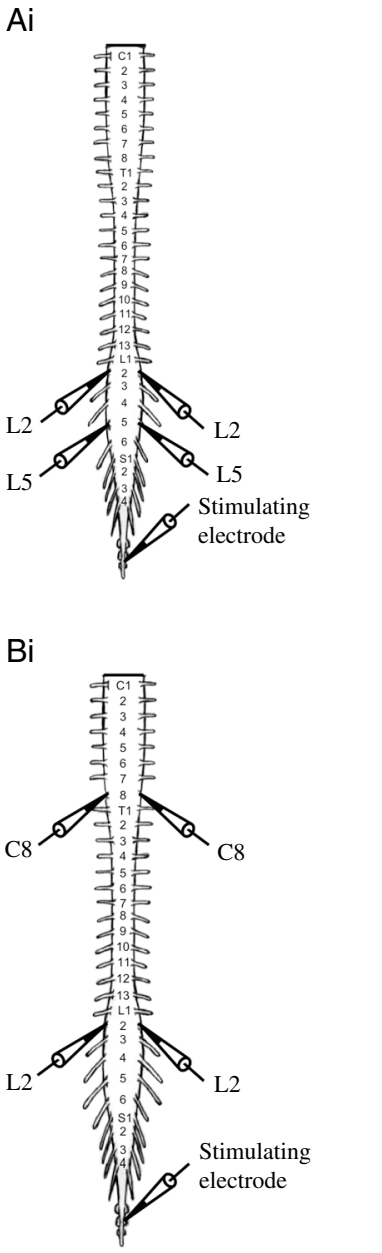

Fig. 2. (Ai) In the neonatal mouse, electrical stimulation (4 Hz, 40 pulses) of the cauda equina is capable of evoking coordinated lumbar locomotor-like activity. (Aii) Rectified and filtered neurogram traces recorded from the second and fifth lumbar ventral roots (L2 and L5, respectively). These traces display both left–right (see L2–L2 and L5–L5 traces) and flexor–extensor (see ipsilateral L2–L5 traces) alternation. This pattern is considered to be a signature of locomotor-like activity. (Bi) A schematic of the recording procedure used to examine coordination between cervical and lumbar segments. (Bii) These digitally rectified and filtered traces show left–right alternation in the cervical roots (C8) that is coupled with the pattern recorded from the L2 neurograms. *l*, left; *r*, right.

It is possible to target the separate progenitor domains since specific transcription factors are expressed in each domain, therefore allowing classes of ventrally located interneurons to be manipulated. This is extremely useful to systems neuroscientists because these cells can be identified in live as well as fixed tissue, and genetically altered mice can be generated that lack classes of interneurons. For example, in mice that lack V0 cells, left–right alternating activity was disrupted (Lanuza et al., 2004). As predicted, flexor–extensor coordination was conserved in these mice since V2 interneurons were still present. Furthermore, at least some V0 cells synapse onto contralateral motoneurons, and a preliminary report suggests that many are rhythmically active during patterned motor output (Gosgnach et al., 2004). These data demonstrate that subpopulations of mainly inhibitory commissural interneurons form part of the circuitry controlling left–right locomotor activity, as in the swim network of the lamprey and *Xenopus* (Grillner, 2003; Roberts et al., 1998). One caveat is that the V0-positive cells are a heterogeneous population since a subpopulation expresses markers for VGLUT2, a glutamate transporter. These excitatory interneurons could be part of the CPG but may not be a part of the circuitry generating alternating left–right patterns of rhythmicity. Indeed, V0 interneurons are not likely to be the only population of commissural interneurons involved in regulating left–right alternation, since preliminary evidence suggests a role for a more dorsally located dI6 population of cells (Gosgnach et al., 2004). Although these new techniques allow us to focus on a subset of interneurons, some of which are involved in rhythmogenesis, it is fair to say that these techniques will need to be combined with intracellular recording approaches to subdivide these groups of interneurons into functional classes of cells (Butt and Kiehn, 2003; Zhong et al., 2006).

An example of combining electrophysiological characterization of neurons with genetic approaches has been published (Wilson et al., 2005). Postmitotic interneurons and motoneurons express transcription factors, such as Hb9, that are involved in the determination of their neuronal fate. Hb9 interneurons exhibit electrophysiological signatures consistent with candidate rhythm-generating interneurons. An exciting possibility is that these neurons form part of the spinal cord CPG kernel. The cells are located in the medial areas of lamina VIII (Fig. 3), where rhythmically active neurons have been found in neonatal rat preparations (Kiehn and Butt, 2003). These cells are rhythmically active during locomotor-like activity, are phase-locked to the pattern recorded from the

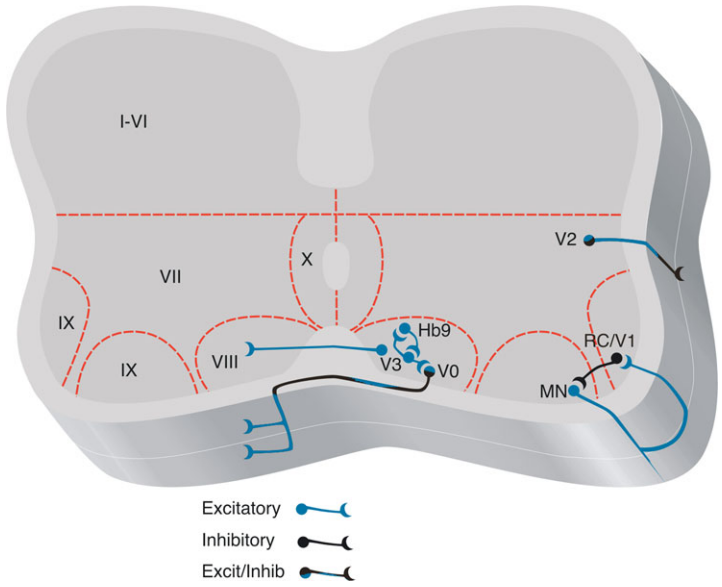

Fig. 3. Genetic techniques can be used to dissect circuits within the spinal cord. Populations of interneurons derived from progenitor cells can be identified in neonatal mice. Schematic shows different populations of cells that can be identified currently. V0 cells are commissural interneurons that consist of both glutamatergic and glycinergic/GABAergic cells. Animals lacking these commissural cells have poor left–right coordination. V1 interneurons are considered to be inhibitory and are a heterogeneous population. At least a portion of them is involved in regulating the speed of the rhythm (Gosgnach et al., 2006). Renshaw cells form part of the V1 population and have been well characterized (Sapir et al., 2004), but the contribution of individual classes of V1 neurons to rhythm generation has not been well established. V2 cells are also likely to be heterogeneous and consist of ipsilateral propriospinal interneurons projecting across several segments. One possibility is that these cells are involved in flexor–extensor coordination. V3 cells are glutamatergic commissural interneurons that may play a role in coordination of patterns. Cells expressing a transcription factor termed Hb9 are present in lamina VIII and exhibit several intrinsic properties thought to be important for rhythm generation (Wilson et al., 2005). Figure adapted from a previously published embryonic schematic (Goulding and Pfaff, 2005).

ventral roots and are glutamatergic (Hinckley et al., 2005; Wilson et al., 2005). Furthermore, voltage-independent membrane oscillations can be recorded in the presence of neuromodulators and tetrodotoxin (TTX; this drug blocks voltage-gated sodium channels and effectively shuts off action-potential-mediated communication between cells). This suggests that a rhythm-generating mechanism exists within these cells. Hb9-positive interneurons also exhibit other intrinsic properties consistent with a role in rhythmogenesis. They possess postinhibitory rebound driven by T-type calcium currents, a common cellular mechanism contributing to alternating oscillatory activity in diverse invertebrate and vertebrate networks (Marder and Calabrese, 1996). Still, it is too early to say that these specific populations of interneurons are necessary and sufficient components of the spinal CPG. To test these ideas, it will be necessary to silence these cells and examine whether locomotor patterns are perturbed. It is essential to discover how the basic rhythm produced by Hb9 cells is converted into muscle-specific locomotor patterns.

Once a population of interneurons is identified, it would be ideal to be able to reversibly alter their excitability to determine whether they affect the function of the CPG. If a population of neurons affects the CPG, the ‘clock’ is changed, and this leads to a change in the frequency of the rhythmic output. These types of experiments would be analogous to invertebrate experiments where disrupting the firing of single interneurons can modulate or reset network activity. Genetic approaches that silence populations of mammalian neurons have been successfully introduced. One approach is to introduce non-native functional receptors into targeted cells. For instance, allatostatin receptors are endogenously expressed in *Drosophila* neurons, where they act *via* G-protein signaling systems to increase the conductance of inwardly rectifying potassium (GIRK) channels (Lechner et al., 2002). If the receptors are artificially expressed in mammalian cell populations they can be activated by allatostatin, thereby activating GIRK channels and reducing the excitability of targeted neurons. For example, in mice designed to express allatostatin receptors only in V1 neurons, it was found that the excitability of V1 populations is reduced following bath application of allatostatin (Gosgnach et al., 2006). During locomotor-like activity in isolated spinal cord preparations of these mice, bath application of allatostatin caused a reversible slowing of the rhythm. The exciting conclusion is that an identified class of inhibitory interneurons contributes to setting the frequency of the rhythm. This is not the only approach, as laser ablation of genetically identified interneurons could also be considered (Buchanan and McPherson, 1995; Liu and Fetcho, 1999). The trick in mammalian systems would be to silence a majority of the targeted class of interneurons to guard against false negative results.

Another technique useful for tracing circuits involves a transynaptic neuronal tracer such as pseudorabies virus (PRV). PRV has been used to trace polysynaptic circuits following injection directly into the CNS or into muscle tissue (Lanuza et al., 2004). This method uses the Bartha strain, an attenuated version of the live virus that is propagated in a retrograde fashion along chains of synaptically connected neurons. However, since the virus invades neurons non-selectively, little could be said regarding the specific classes of neurons infected. To circumvent this limitation, De Falco and colleagues designed a modified PRV virus that will only replicate in cells designed to express Cre recombinase (DeFalco et al., 2001). After replication, the modified PRV expresses green fluorescent protein (GFP) in the targeted cells only. This tool has several advantages since, for example, mice expressing Cre recombinase in selected classes of interneurons (V0–3) could then be combined with the modified PRV tracing of motoneurons. This would allow discrete tracing of classes of

premotor interneurons synapsing onto motoneurons, which would facilitate the mapping of network connectivity within the spinal cord. While these types of tracing studies do not reflect the dynamic nature of network connectivity, they will be helpful to electrophysiologists interested in selecting populations of cells that contribute to rhythmogenesis.

Another successful approach involves identifying mutants exhibiting a deficit in gait and subsequently examining the changes in network circuitry and function. For example, EphrinB3 is one of many guidance cue molecules in the nervous system and is expressed in the midline of the spinal cord, where it normally repels growth cones containing the EphA4 receptor (Palmer and Klein, 2003). Recent data suggest that spinal neurons that express EphA4 receptors in normal mice are ipsilateral excitatory cell types that potentially contribute to rhythmogenesis (Butt et al., 2005). Accordingly, EphA4 and EphrinB3 mutant mice produce a characteristic hopping gait (Kullander et al., 2003; Pearson et al., 2005). The lack of EphA4 receptors or its ligand EphrinB3 in the mutant allows for the aberrant growth of excitatory commissural interneurons that lead to the hopping rather than alternating gait. In our lab, we have recently studied a mutant mouse that exhibits similar behavior, although the hopping deficit is confined to the hindquarters (Whelan and Madriaga, 2004). Like the EphA4 mouse, the deficit appears to be due to a change in spinal cord circuitry since the hopping pattern could be elicited using isolated *in vitro* spinal cord preparations. The spontaneous mutation is on the *hop* gene localized to chromosome 6. The cause of the hopping deficit in this mutant is unknown. However, there is a marked reduction in the production of *braf*, a member of the *raf* family of serine/threonine kinases, which is an important regulatory element in the extracellular signal-related kinase (ERK) signaling pathway (Wojnowski et al., 1998). The ERK pathway, as a part of the mitogen-activated protein kinases (MAPK) signaling cascade, is significant in the netrin-1-mediated guidance of commissural axons (Forcet et al., 2002). Although speculative at this stage, a reduction in *braf* may reduce the number of commissural axons in the *hop* mutant mice (Wojnowski et al., 1998). However, much work needs to be done to correlate this deficit with a specific spinal circuit. As in the case for many mutants, an important caveat is that the affected gene or genes may have multiple combinatorial downstream effects. Nonetheless, both the *hop* and the EphA4 mouse may provide a fascinating window into alterations in CPG structure that led to the evolution of the kangaroo mouse and rat.

Overall, genetic techniques have greatly expanded the types of experiments that can be performed, but the specificity of these techniques will need to be carefully validated using a combination of electrophysiological and selective ablation techniques (Kiehn and Butt, 2003). We have just started to mine the potential of genetic techniques, and in the future we can expect that it will be possible to target classes of interneurons that form specific locomotor and reflex circuitry. It is noteworthy that transcription factors (e.g. Fezl) are being identified that are expressed in discrete descending tracts such as the corticospinal tract (Molyneaux et al., 2005). Thus, in the future, a greater range of tools for manipulating the excitability of spinal circuits and descending neurons that project onto spinal CPGs should become available.

New directions and conclusions

New interventions that target and tune specific spinal circuits would potentially improve the functional outcome for patients with spinal cord injury. The good news is that the tools necessary to move beyond the black box concept of mammalian spinal CPGs are finally being developed. The ultimate test of causality would be if CPG circuits could be reversibly switched on and off during walking in conscious animals. ‘Remote control’ of escape circuits has been recently achieved in intact fruit flies, where unfocused laser light shone over the genetically modified flies uncaged a ligand in a lock-and-key activation system, with the ligand receptors expressed on selected neurons in the escape circuit (Lima and Miesenbock, 2005). In mammals, a variant of this experiment could be performed in which allatostatin (Lechner et al., 2002; Gosgnach et al., 2006) is intrathecally applied to activate GIRK channels in selected spinal cord interneurons. This would allow reversible reduction in the excitability of classes of interneurons in walking mice. Resultant changes in gait could then be quantified using kinematic and EMG recording techniques (Pearson et al., 2005). One potential issue is that, since allatostatin activates G-protein-coupled receptors, some desensitization would be expected over time. Nevertheless, this tool has a great deal of potential.

The use of optical techniques, such as calcium imaging, has yielded valuable information on spinal network dynamics (Bonnot et al., 2002b; Nakayama et al., 2002). Multiphoton techniques allow visualization of neurons deep within spinal cord tissue (Bonnot et al., 2005; Kerschensteiner et al., 2005). These techniques, combined with genetic approaches, allow optical recordings to be obtained from populations of neurons *in vivo* (Mizrahi et al., 2004). For example, it is possible to intracellularly stimulate a cortical cell and visually identify which cells are activated using optical techniques (Cossart et al., 2005; Ikegaya et al., 2005). Extending these techniques to the spinal cord could reveal exciting new data about the dynamic recruitment of selected classes of interneurons during locomotor activity.

In closing, it was not that long ago that the possibility of dissecting specific classes of interneurons that form part of the mammalian CPG would have been considered improbable. Over the past five years, new tools and approaches have created a new sense of optimism that we will be able to decipher complex spinal networks that generate terrestrial locomotion in mammals.

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