Review

Shining light into the black box of spinal locomotor networks

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Rhythmic activity is responsible for numerous essential motor functions including locomotion, breathing and chewing. In the case of locomotion, it has been realized for some time that the spinal cord contains sufficient circuitry to produce a sophisticated stepping pattern. However, the central pattern generator for locomotion in mammals has remained a 'black box' where inputs to the network were manipulated and the outputs interpreted. Over the last decade, new genetic approaches and techniques have been developed that provide ways to identify and manipulate the activity of classes of interneurons. The use of these techniques will be critically discussed and related to current models of network function.

Keywords: spinal cord; locomotion; central pattern generator; mouse; networks

1. INTRODUCTION

Nearly 100 years ago, Graham-Brown published a seminal article describing his observations on locomotion in spinal cats (Graham-Brown 1911). Using a set of elegant experiments, he demonstrated that circuitry within the spinal cord is sufficient to produce coordinated rhythmic movements of the hindlimbs. He postulated that a set of flexor and extensor centres (half-centres) connected by mutually inhibitory connections produced the rhythmic stepping pattern.

Over the years, this model has been used as a framework for understanding how locomotor activity is produced in a wide variety of vertebrate species. Naturally, it has been revised and expanded over the years, but the essence of Graham-Brown's original half-centre hypothesis continues to be relevant today. As pointed out in an interesting historical review, Graham-Brown's summary of his 1911 paper nicely summarizes the *current* conceptual framework regarding the modulation and generation of stepping (Stuart & Hultborn 2008). As a field, we have provided support for Graham-Brown's ideas using a diverse range of vertebrate species such as the lamprey, tadpole, turtle, cat and rodent (Jankowska *et al.* 1967*b*; Lundberg 1979; Roberts *et al.* 1998; Stein *et al.* 1998; Roberts 2000; Grillner 2003; Kiehn 2006; Stuart & Hultborn 2008). The problem then and now is how to identify and selectively silence classes of interneurons that we suspect form part of the locomotor network. Genetic tools are providing a sophisticated range of methods to trace neural circuits, and identify and manipulate the activity of classes of interneurons (Gordon & Whelan 2006; Kiehn 2006; Luo *et al.*

2008). The impact of these tools on neuroscience and spinal cord networks in particular cannot be overstated. On the other hand, the initial flurry of studies while technically outstanding have not yet provided a conceptual advance that have leapfrogged us beyond that articulated by Graham-Brown. Most probably, this is because networks, no matter where they are located, are difficult to understand (Parker 2010; Selverston 2010). To fully appreciate their operation, a multi-pronged approach combining behavioural, genetic, modelling, intracellular and imaging techniques needs to be applied.

2. OUTLINE OF THE PROBLEM

Peter Getting, among others, outlined a set of steps required to understand a rhythmic network (Getting 1989; Selverston 2005; Yuste *et al.* 2005). This started with an analysis of the output of the network and ended with the ability to successfully model the network. In between, it was argued that we need to understand how the building blocks of the network interact to produce the behaviour. This means that we need to understand the connectivity between the building blocks of the network. However, this by itself is not enough, since networks are not hardwired and can flexibly reconfigure by adjusting synaptic and intrinsic properties of the neurons (Marder & Bucher 2001). To build a realistic model of networks, the classes of interneurons need to be identified along with their connections, as well as their intrinsic and synaptic properties. Even in comparatively simple invertebrate networks, this is easier said than done. In a provocative study that modelled a three neuron simplified stomatogastric ganglia network, it was found that over 3000 different weightings of intrinsic and synaptic properties could account for the output of the network (Prinz *et al.*

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2004). This suggests that even if the output of the network appears to be constant, the network connectivity does not have to be the same to produce a given behaviour (Prinz 2010). Clearly this issue has broad implications when examining spinal mammalian networks, which consist of thousands of neurons.

The additional complexity of terrestrial mammalian networks is required to control over 80 muscles to produce fluid stepping behaviour. Acceleration and deceleration of limbs must be precisely timed to produce a seamless gait pattern (Grillner 1981). The spinal network is constructed in such a way that the output is both robust yet modifiable so that it accommodates volitional and reflexive changes to the basic pattern. Furthermore, during development, as limbs and muscles grow and the animal gets heavier, spinal networks are capable of adjusting to the changing biomechanics of the system. For example, human males increase body weight by over 700 per cent during development, yet the basic gait continues to be effortlessly produced.

Determining how the mammalian central pattern generator (CPG) is constructed to accommodate these demands is a fascinating and demanding area of research. Genetic approaches are providing new approaches at a rapid pace, especially in the field of optogenetics (Deisseroth *et al.* 2006). It is now possible to design experiments that selectively and rapidly turn on and off populations of interneurons using light (Airan *et al.* 2007). These tools should prove to be useful to test whether a class of interneurons is necessary or sufficient for spinal network function. The terms necessity and sufficiency are commonly used in the description of networks and are not interchangeable. A class of interneurons is necessary for CPG function if their removal from the circuit eliminates some definable aspect of the rhythmic output. For example, if the ability to produce left–right alternating activity is abolished when a class of interneurons is removed, then that class would be deemed necessary for that behaviour. Similarly, if blocking a class of interneurons eliminated rhythmic activity, then it would suggest that activation of the class was necessary for operation of the CPG. That said, one has to be cautious in interpreting the blockade of activity as follower interneurons rather than interneurons comprising the rhythm generator may be affected. A careful analysis of the pattern can resolve these issues (McCrea & Rybak 2008).

On the other hand, selectively activating a class of interneurons may be sufficient to produce the rhythm. However, it does not imply that the class is *necessary*. This is because there may be redundant classes of interneurons, each of which may be sufficient to evoke the behaviour. It is essential, therefore when designing tests of necessity and sufficiency to define the behaviour.

3. BRIEF HISTORY OF MAMMALIAN NETWORKS

Like many areas of science, progress in the understanding of spinal networks has been a function of conceptual advances, availability of technical tools and historical circumstances. Conceptually, major advances were made by Graham-Brown (Graham-Brown 1911; Stuart & Hultborn 2008). Following his discovery, advances were slow, probably because of a lack of recognition of the importance of this early work (Stuart & Hultborn 2008). Part of this was because of an increased focus on recording intracellular potentials, with the exciting advance of John Eccles making the first intracellular recording from a mammalian neuron—the motoneuron (Brock *et al.* 1952). In the 1960s, renewed interest in mammalian networks came from the work of Anders Lundberg and colleagues in Sweden who found that treatment of spinalized cats with L-DOPA could allow high-threshold afferents to activate flexors and then extensors in a mutually inhibitory manner (Jankowska *et al.* 1967*a,b*). These were the first set of studies to provide evidence in support of Graham-Brown's half-centre hypothesis. This work by Lundberg, his colleagues and trainees led to a renaissance in the field of spinally generated locomotion (Stuart & Hultborn 2008). Around this time, the concept of the CPG first appeared in the literature (Wilson & Wyman 1965; Stuart & Hultborn 2008). The CPG is defined as a set of interneurons that are capable of producing rhythmic output without phasic input from afferent or descending projections. Later on, work using L-DOPA would show that alternating flexor and extensor locomotion could be generated in cats (Grillner & Zangger 1975, 1979). By 1981, a large body of work had been amassed on the operation of networks in cats. This was summarized in a comprehensive review by Grillner (1981). Considering data from work on the cat, Grillner proposed the unit oscillator model of pattern generation (Grillner 1981). A unit oscillator is a simple network controlling movement around a single joint such as the ankle or knee. The unit oscillator model could account for complex behaviours such as walking by connecting multiple unit oscillators through excitatory and inhibitory connections. The advantage of this system is that it is extremely flexible, and different behaviours can be produced by altering the coupling between the sets of unit oscillators. While we have focused on the centrally generated pattern in the above discussion, it is well recognized that sensory input plays a fundamental role in the sculpting the timing and pattern of the rhythm (Rossignol 1996; Whelan 1996).

Around the late 70s and early 80s, there was some agreement in the field that the cat spinal network was too complex to be understood fully with the tools available (Cohen & Wallen 1980). The cat spinal network represented a black box in which inputs were delivered and outputs analysed. While much work had been performed identifying interneurons receiving group I and II afferent input (Jankowska 2001), the problem was how to identify interneurons that comprised the circuit itself. These neurons do not necessarily have to receive afferent input and are not clustered into a nucleus. Lest the cat be discounted, it must be remembered that today work from the cat still provides the most extensive analysis of identified interneurons of any mammalian model (Lundberg 1979; Jankowska 1992).

Around this time, the examination of spinal loco-motor networks began to turn to vertebrates that produced locomotor behaviour with fewer classes of interneurons. A useful example is the development of the lamprey as a model preparation where the relatively small number of cells allowed the identification of functional classes of interneurons that comprise the swim CPG (Cohen & Wallen 1980; Poon 1980; Buchanan 1982, 2001; Buchanan & Cohen 1982; Grillner 2003; cf. Parker 2006). This led to a series of studies and new preparations including the turtle swim and scratch CPG (Stein *et al.* 1998), the *Xenopus* swim CPG (Roberts *et al.* 1998, 2008) and the zebra-fish escape circuit (Fetcho *et al.* 2008). The use of these preparations has led to several seminal findings that have advanced the field and that have been ably described in several reviews (Roberts *et al.* 1998, 2008; Stein *et al.* 1998; Buchanan 2001; Fetcho *et al.* 2008). At the same time, CPG work using the cat was not dormant, with interesting findings emerging on the interaction of reflex pathways and the control of stepping (Forssberg *et al.* 1975, 1980; Grillner & Rossignol 1978; Lundberg 1979; Duysens & Pearson 1980; Barbeau & Rossignol 1987).

Toward the end of the 1980s, there appeared a set of papers from Jack Feldman's laboratory demonstrat-ing the ability of an isolated rat spinal cord preparation to generate locomotor-like behaviour *in vitro* (Smith & Feldman 1987; Smith *et al.* 1988). This preparation, which was inspired by earlier work by Suzue (1984), allows ease of access to the spinal cord, manipulation of the extracellular milieu and use of lesioning meth-odologies, which greatly aided the examination of spinal networks. Yet, despite the subsequent popu-larity of this preparation, the mammalian CPG remained a 'black box', even under *in vitro* conditions.

Starting in the late 90s, the mouse started to gain popularity as a model preparation for the study of locomotion (Bonnot & Morin 1998; Bonnot *et al.* 1998; Jiang *et al.* 1999; Whelan *et al.* 2000). The adop-tion of the mouse for studying mammalian network function opened the door to genetic approaches. An important event was the collaboration of deve-lopmental neurobiologists with electrophysiologists investigating network function (Kullander *et al.* 2003; Lanuza *et al.* 2004; Miles *et al.* 2004; Gosgnach *et al.* 2006; Zhang *et al.* 2008; Crone *et al.* 2009). This capi-talized on the discovery by developmental biologists that classes of motoneurons and interneurons could be defined by expression of progenitors and combina-torial expression of transcription factors (Jessell & Sanes 2000). During the early 2000s, it is fair to say that the entire field was energized by the potential impact of these genetic approaches (Kiehn & Kullander 2004). The potential cannot be overstated since tools now exist that can label interneurons that are candidates to be part of the CPG (Lanuza *et al.* 2004; Wilson *et al.* 2005; Gosgnach *et al.* 2006; Crone *et al.* 2008; Zhang *et al.* 2008; Goulding 2009). That said, clearly deciphering the structure of the spinal CPG using genetic approaches is going to be a complex task.

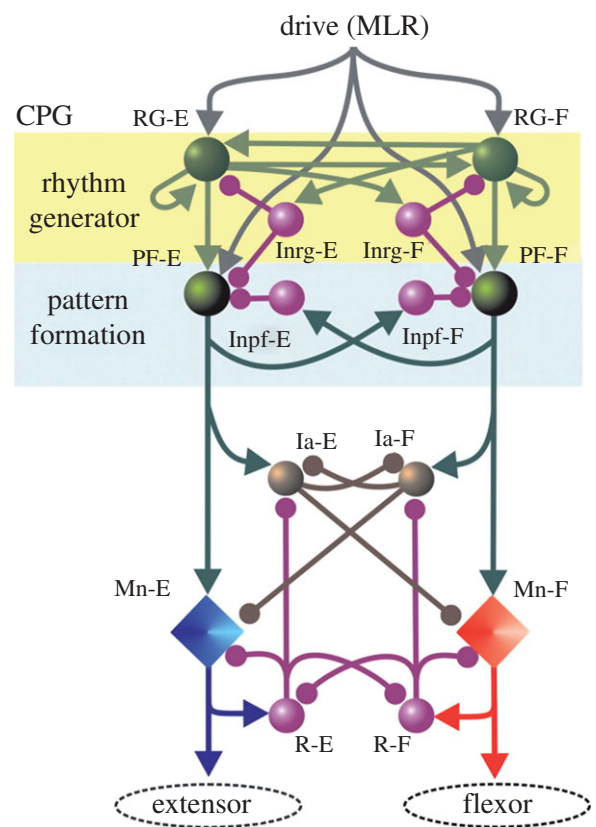Right now, there are two dominant theories of CPG function. The first, as discussed earlier, is the unit

Figure 1. Schematic of model by Rybak & McCrea. The populations of interneurons are indicated by spheres, while the motoneurons are represented by diamonds. This three-layer model consists of a rhythm-generating layer of extensor (RG-E) and flexor (RG-F) interneurons. Both populations have recurrent excitatory connections (see also figure 2). These interneurons in turn receive mutually inhibitory input (Inrg cells). The drive projects to a pattern formation layer (PF), which acts through mutually inhibitory connec-tions (Inpf cells) to sculpt the pattern, which is then output to the extensor and flexor motoneurons. The final output of the motoneurons is modulated by a final layer of Ia inhibitory interneurons (Ia-E, Ia-F) and Renshaw cells (R-E, R-F). Arrows indicate excitatory drive, while the filled circles indicate inhibitory drive. Reproduced with permission.

oscillator hypothesis (Grillner 1981). The other is a three-layer model that builds on the original half-centre model (Lafreniere-Roula & McCrea 2005; Rybak *et al.* 2006*a*; McCrea & Rybak 2008). The first layer is pro-posed to consist of a distributed network consisting of excitatory interneurons interconnected which form mutually inhibited half-centres. This first layer is the rhythm generator whose drive is then propagated to the pattern formation layer, which provides the specific drive that is sent to a population of motoneurons innervat-ing a particular muscle (figure 1). The third layer is a set of mutually inhibitory classes of Ia inhibitory interneurons that also receive input from Renshaw cells. These inhibi-tory Ia interneurons do not contribute to rhythmogenesis but do contribute to the shaping of overall motoneuronal output. There is both experimental and modelling data suggesting that this multiple-layer model can explain many of the behaviours produced (Lafreniere-Roula & McCrea 2005; McCrea & Rybak 2007, 2008; Endo & Kiehn 2008).

4. THE DREAM

The mammalian CPG still remains a black box, but new tools are providing methods to dissect the networks. Before delving into what technology is available, we should pause and review what would be a dream scenario for researchers of spinal cord networks. We would like to have a set of tools that would allow us to test necessity and sufficiency for sets of identified candidate interneurons that may form part of locomotor networks. Ideally, these tools would be specific enough to allow targeting of Renshaw cells, Ia interneurons, commissural interneurons (CCINs) and so on. Under ideal conditions, one can conceive of a switchboard where one could reversibly silence sets of neurons to examine whether the class of interneurons was necessary for network function. At the same time, tests of sufficiency would involve selective activation of the targeted classes to examine whether they alone could elicit episodes of rhythmic activity or influence activity in predictable ways. Ideally, we would be able to identify a single class of conditional pacemaker cells that form the kernel of the network (Marder & Bucher 2001). These pacemaker cells would be responsible for generating the timing of the rhythm and would project to pattern-generating interneurons that sculpt the pattern before in turn projecting onto motoneurons (McCrea & Rybak 2008). Distinct classes of pattern-generating interneurons could be identified and reversibly inactivated to influence the pattern but not the timing of the rhythm. Recent developments indicate that we are getting close to this dream of having a genetic-based switchboard where we can turn on and off selected classes of interneurons (Luo et al. 2008). The outlined scenario is perhaps the most tractable from an experimental point of view. While we still do not know the structure of the mammalian network, it is well known that other models can account for CPG output. Therefore, data collected using new technologies will have to be carefully interpreted with no a priori bias for one model versus another.

5. CURRENT APPLICATIONS OF GENETIC TECHNOLOGIES

Several years ago, Tom Jessel and colleagues showed that neurons could be clustered into distinct classes based on the expression of transcription factors in progenitor cells (Jessell & Sanes 2000). During early development, 11 progenitor cell classes lead to the production of postmitotic cells by embryonic day 11 in mouse. Six of these classes of interneurons contribute to the formation of the spinal circuitry contributing to CPG function and motoneuronal output (Goulding & Pfaff 2005; Goulding 2009). Each of these early interneuronal classes has unique anatomical and transmitter phenotypes (figure 2). The so-called V0 interneurons project across the ventral commissure and ascend for two to four segments (Lanuza et al. 2004). They are thought to project onto Renshaw cells, Ia inhibitory interneurons, and motoneurons. The V0$_D$ are GABAergic/glycinergic cells that contribute to the control of left–right alternating activity (Lanuza et al. 2004). On the other hand, the V0$_V$ class is glutamatergic neurons (Goulding 2009) that do not appear to contribute to locomotor coordination

(Lanuza et al. 2004). The V1 class of neurons is restricted to the ipsilateral side of the spinal cord in lamina VII and are inhibitory (Sapir et al. 2004; Alvarez et al. 2005). V2 neurons can be subdivided into V2a and V2b classes (Peng et al. 2007) which are excitatory and inhibitory, respectively (Al-Mosawie et al. 2007; Lundfald et al. 2007). V2 cells do not cross the midline and descend ipsilaterally across several segments. Recent studies indicate that the excitatory V2a interneurons have diverse effects on network output, including left–right coupling, speed, amplitude and regularity of the rhythm (Crone et al. 2008, 2009). Interestingly, their effects on left–right coupling are probably due to projections onto CCINs since V2a cells do not project across the midline (Crone et al. 2008). The role for the V2b class has not been determined, but since the neurons project caudally and are inhibitory, it could contribute to the control of reciprocal inhibition between flexors and extensors (Goulding 2009). The V3 class of interneurons is a mixed class of cells that project both ipsi- and contralaterally (Zhang et al. 2008). However, in contrast to V0 interneurons, they do not appear to contribute to left–right coordination, although they do contribute to the regularity of the rhythm (Zhang et al. 2008). In addition, there is a dorsally identified class of inhibitory interneurons that are derived from dorsally located progenitors termed the dI6 population (Muller et al. 2002). These cells project across the midline and are probably involved in controlling the pattern of locomotion (Goulding 2009).

Each of these classes of interneurons express a unique set of transcription factors that allows the classes to be silenced either permanently or transiently and the effects on locomotion assessed (Lanuza et al. 2004; Gosgnach et al. 2006; Crone et al. 2008, 2009; Zhang et al. 2008). Alternatively, their synaptic and intrinsic properties can be assessed when the neurons are made to express fluorescent proteins (XFPs) by recording from them using electrophysiological or imaging techniques in live slice or isolated spinal cord tissue (Lanuza et al. 2004; Wilson et al. 2007; Dyck & Gosgnach 2009). Different classes of cells can be targeted using different XFPs (Wilson et al. 2007), potentially allowing targeted imaging of circuits as they turn on and off during rhythmic activity. Furthermore, classes of interneurons can be inactivated using pharmacological approaches when non-natively expressed channels are inserted (Lechner et al. 2002; Gosgnach et al. 2006). This overcomes a major obstacle of single-cell recordings that only examine the activity of a single neuron. It also allows tests of necessity and sufficiency to continue across classes of spinal cord interneurons. Therefore, inferences regarding their function can be determined by examining the effects of selective and reversible activation or inactivation of ventral horn interneurons.

Naturally, there are caveats to the use of these techniques, which limit interpretation of the data. The chief issue is that the changes in the output of the network following silencing of the targeted class of interneurons are often difficult to interpret. For example, recently it was shown by Martyn Goulding's

group that the V1 population of inhibitory inter-neurons was involved in setting the timing of the rhythm. The study was a tour de force, and notably included for the first time in our field a test of necessity. The authors expressed a non-native allatostatin receptor in the V1 class of interneurons. Allatostatin is a peptide that was identified in *Drosophila* (Birgul *et al.* 1999) and leads to activation of GIRK channels that hyperpolarizes the neuron (Lechner *et al.* 2002). When the receptor for allatostatin is introduced into a class of neurons, one can inactivate a defined population of cells by simply bath applying allatostatin (Gosgnach *et al.* 2006; Zhang *et al.* 2008). This approach can also be used in adult mice by introducing allatostatin intrathecally and examining changes in the stepping pattern (Zhang *et al.* 2008). Using this approach, the authors found that inactivation of the V1 inhibitory cells led to a slowing of a rhythm. While this study showed that these cells contributed to the timing of the rhythm, they were not essential (see later discussion of distributed networks). At present, it is difficult to draw conclusions regarding the role of individual classes of V1 interneurons such as IaINs and Renshaw cells. Presumably greater precision will be required to make definitive conclusions regarding the role of the V1 class of interneurons in the timing and patterning of locomotion since at present 75 per cent of the interneurons in this class remain unclassified (Goulding 2009). While there are some ambiguities and difficulties to be resolved, the tools being developed, such as the one discussed (Gosgnach *et al.* 2006), represent an enormous movement forward for our field.

With the breathtaking progress in the genetic field, network electrophysiologists have numerous new targets to investigate using the mouse. What would make the job easier is more electrophysiological data on afferent connectivity within the spinal cord of the mouse. Most of the electrophysiological classification of interneurons has been accomplished using the adult cat (Jankowska 1992, 2001). We need to examine whether similar connections are made in the neonatal and adult mouse. Indeed, recent evidence in the mouse is starting to turn up interesting differences in connectivity between the mouse and the cat (Mentis *et al.* 2006).

Apart from the Renshaw cells, which are well characterized in the mouse (Mentis *et al.* 2005, 2006; Nishimaru *et al.* 2005, 2006), interneurons in the cat spinal cord were identified by relatively selective activation of classes of afferents (Jankowska 1992, 2001). In developing rodents, it is not as straightforward to selectively activate classes of afferents, and as a consequence, the necessary studies correlating classes of afferent-identified interneurons have not been performed. A problem is that the strategy of using low to high levels of electrical stimulation in the cat to target group I muscle afferents is not very effective in the neonatal mouse or rat. Therefore, it will be necessary to activate muscle spindles (group Ia/II afferents) using piezoelectric vibrators (Iizuka *et al.* 1997) and miniature muscle pullers (group Ib afferents) to activate Golgi tendon organs. Molecular approaches will probably be developed that allow the

selective activation and inactivation of classes of muscle afferents, much as the case for pain afferents (Caterina *et al.* 1997; Mandadi *et al.* 2009), but this still remains to be resolved.

A more serious issue is that these approaches will not work for interneurons that do not receive afferent input and form part of the CPG network. Therefore, these classes of interneurons will need to be activated and inactivated using genetic approaches one class at a time to determine their effects on network output. It is sobering to think of doing this when the structure of the 'clock' or kernel of the CPG is probably distributed (figure 3) across multiple segments (Kjaerulff & Kiehn 1996; Cowley & Schmidt 1997; Kremer & Lev-Tov 1997). It is still a matter of debate whether the different patterns are due to multiple unit oscillators or alternatively due to a separate patterning layer (Grillner 1981; Rybak *et al.* 2006*a,b*; McCrea & Rybak 2007, 2008). Regardless of the outcome of this debate, the fact remains that multiple neuronal classes probably contribute to the formation of the kernel of the CPG. The closest the field has come to identifying a class of cells that may form part of the kernel of the CPG are the Hb9 interneurons (Hinckley *et al.* 2005; Wilson *et al.* 2005). Hb9 is a transcription factor that is expressed in a subclass of ventrally located interneurons that fire in phase with rhythmic activity from the same segments. They also possess conditional pacemaker properties (Wilson *et al.* 2005; Han *et al.* 2007) along with other intrinsic properties such as postinhibitory rebound that make them ideal candidates for interneurons that form part of the CPG kernel (Wilson *et al.* 2005). However, their role in generating locomotion remains controversial for several reasons (Kwan *et al.* 2009). First, they tend to fire after the onset of motoneuronal activity in L2 segments. Second, they fire few spikes during a bout of locomotion. Third, they are not present in caudal lumbar segments, even though alternating activity can be evoked from isolated caudal lumbosacral segments. Finally, Hb9 interneuronal firing tends to be suppressed when locomotion is evoked by electrical stimulation compared with pharmacological activation. While these observations tend to rule out a role for Hb9 interneurons in forming a kernel of critical pacemaker neurons, they do not rule out a role for Hb9 interneurons being a component of a distributed network of cells that mutually excite each other. Numerous studies show that other cells share conditional oscillatory properties and it would be interesting to examine the firing behaviour of these cells in relation to ventral root bursting behaviour. Ultimately, evidence for a role for Hb9 interneurons awaits tests of necessity and sufficiency.

Rather than a single pacemaker class within the spinal cord, a more likely scenario is that we will discover that multiple classes of genetically identified interneurons form the kernel of the CPG (figure 3). Indeed, the literature already shows evidence for conditional oscillations being more the norm rather than the exception in ventrally located interneurons in lamina VIII and VII in the rodent (Hochman *et al.* 1994; Han *et al.* 2007; Tazerart *et al.* 2007). A distributed network is not great news for experimenters since
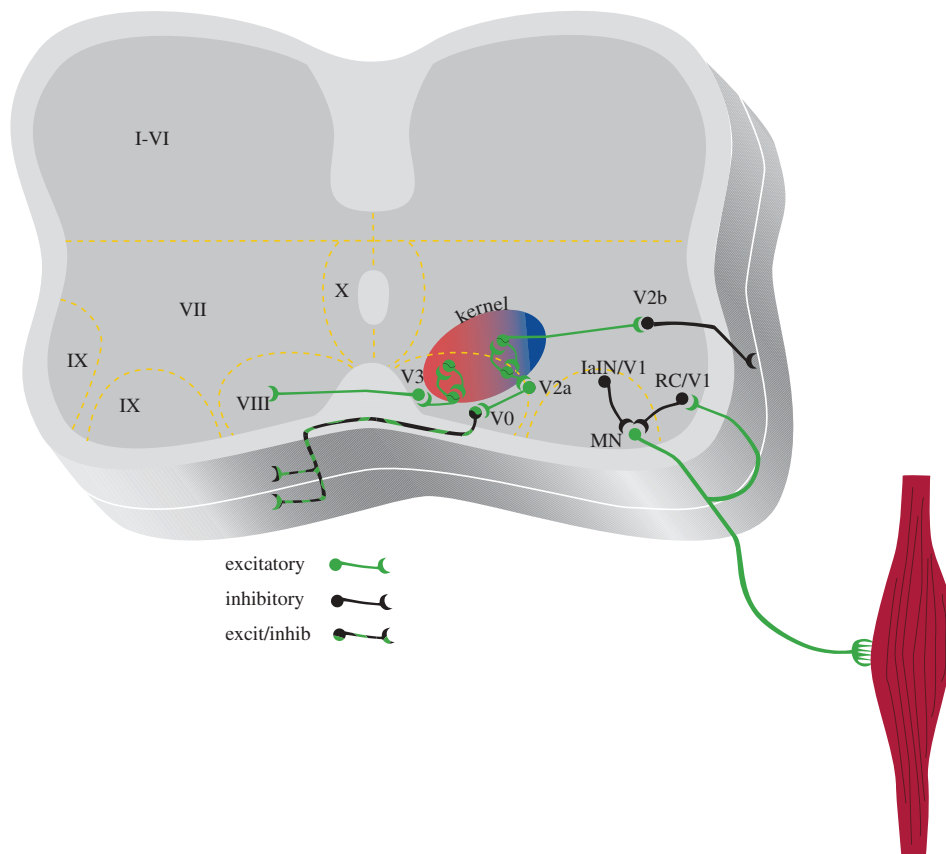
Figure 2. Genetic techniques can be used to dissect circuits within the spinal cord. Populations of interneurons derived from progenitor cells can be identified in neonatal mice. Schematic shows different populations of cells that can be identified currently in neonatal mouse preparations. V0 cells are CCINs 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. Renshaw cells and Ia inhibitory interneurons (IaINs) form part of the V1 population and have been well characterized, but the contribution of individual classes of V1 neurons to rhythm generation has not been well established. V2 cells are also heterogeneous and consist of a population of excitatory interneurons (V2a) that project ipsilaterally and are involved in the regulation of left–right coordination. Another population of inhibitory interneurons possibly projects caudally and regulates flexor–extensor alternation. V3 cells are glutamatergic CCINs that may play a role in coordination of locomotor patterns. Interneurons that belong to the kernel that generates the timing pattern have not been conclusively identified. See text for references.

no one class of interneurons can be reversibly inactivated and found to be necessary for the rhythm. More likely, what would occur is graceful degradation of the rhythm. Graceful degradation is a term commonly used in the design of distributed computer networks (Randell *et al.* 1978). It refers to the ability of the network to maintain limited functionality even if large portions of it are destroyed or rendered inactive. In essence, it is a design to prevent catastrophic failure if elements of the network fail. Perhaps the best known implementation of this system is the worldwide web. This analogy is useful when thinking about a network as important as the locomotor network. The prediction would be that the rhythm would slow down or tend to become irregular as elements of the distributed network were silenced. This is often what is observed when lesion studies or split-bath techniques are used to inactivate areas of the spinal cord (Kjaerulff & Kiehn 1996; Cowley & Schmidt 1997; Kremer & Lev-Tov 1997). To test this idea using genetics, multiple classes of

interneurons will have to be turned off independently of each other. For the sake of argument, let us say that there are eight classes of cells that we hypothesize form the kernel of the CPG. If class 1 + class 2 are turned off and the rhythm stops then it would argue that both of these classes are necessary for rhythmogenesis. However, if class 5 + class 8 are inactivated, or any other combination, and the rhythm is blocked, then it suggests that a distributed network exists. It can be appreciated that to test all combinations would be a time-consuming task. A key concern is carefully controlling for a false negative. Let us say we find evidence that both classes 1 and 2 are necessary for rhythmogenesis. However, this may be simply due to a reduction in the net excitability of the network owing to the removal of these elements. In a distributed network, it should be possible to increase the excitability of the network by increasing the concentration of rhythmogenic drugs (Kjaerulff & Kiehn 1996; Kremer & Lev-Tov 1997) or by increasing the electrical stimulation intensity. Another feature of distributed
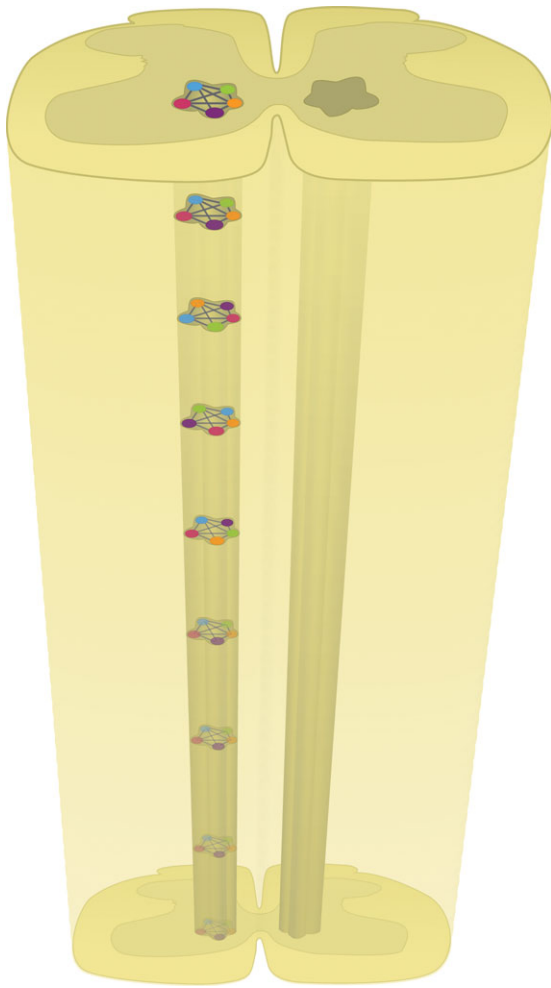
Figure 3. The rhythm generator kernel is probably distributed throughout the spinal cord. This diagram shows different classes of mutually excitatory populations of interneurons that form one part of a rhythm generator (RG) half-centre. The different classes comprising the single RG can be interconnected in different ways throughout the network. In this system, deletion of a single class of interneurons would not be expected to completely block the rhythm.

networks is that they often exhibit network homeostasis (Maffei & Fontanini 2009). Network homeostasis has been observed in several species including the spinal cord of the embryonic chick (Chub & O'Donovan 1998; Tabak *et al.* 2001). Therefore, even after the rhythm is blocked, compensation within the network can result in the return of rhythmicity. What this means is that appropriate controls that allow for extended silencing of each interneuronal population should be completed.

So far we have discussed the genetic manipulation of classes of interneurons and the subsequent modification of network function. In all likelihood, these *bottom-up* approaches (reverse genetic screen) will need to be married with a *top-down* or forward screening strategy that targets spontaneous mutations, some of which produce interesting behavioural phenotypes. Essentially, this approach works in the opposite direction. The mutation that produces the behaviour needs to be mapped to a particular locus on the genome that corresponds to a gene or a set of genes. Then these

genes need to be ablated in normal mice to see if the same behaviour can be reproduced. An example of this approach is the use of mice lacking a receptor EphA4 or its ligand EphB3 that normally functions to repress certain classes of developing excitatory interneuronal axons from crossing the midline (Kullander *et al.* 2003). These mice exhibit a hopping-like locomotor behaviour instead of normal alternating stepping. This behaviour was thought to be caused by excitatory glutamatergic interneurons that normally innervate neurons ipsilaterally, which instead made aberrant connections with the contralateral network. The additional excitatory commissural drive appeared to overwhelm the drive from inhibitory CCINs. However, recent evidence suggests that the other possibility, namely that inhibitory CCINs are reduced, could also be responsible (Restrepo *et al.* 2009*a*).

Similar types of approaches have been used with success in other species, such as the zebrafish (McLean & Fetcho 2008), to identify candidate interneurons that contribute to rhythmogenesis. An excellent example of this approach was published recently whereby a screening approach was used to isolate a particular cell type (Wyart *et al.* 2009). The authors used an optogenetic approach whereby they inserted a light activated glutamate subunit into the cells they wished to examine. Using this approach, they were able to verify that photoactivation of a specific class of cells was sufficient to induce locomotion. Furthermore, they were able to selectively reduce synaptic transmission from these classes of cells and correlate this to a marked reduction in the frequency of locomotion. These results are exciting since to the best of my knowledge it represents the first time that genetic techniques have been used to test sufficiency and necessity in a *homogeneous* class of vertebrate spinal cord neurons.

6. FUTURE APPLICATIONS OF TECHNOLOGY IN SPINAL CORD SYSTEMS

Assuming the CPG kernel is distributed, I suspect that genetic approaches will be most beneficial when used to examine the pattern-generating layer. A good example is the CCINs. These CCINs connect networks on each half of the spinal cord and are responsible for coordinating left–right and flexor–extensor limb coordination (Kiehn 2006). Several classes of CCINs have been identified using both genetic (Lanuza *et al.* 2004; Zhang *et al.* 2008; Goulding 2009) and anatomical approaches (Butt *et al.* 2002; Stokke *et al.* 2002; Bannatyne *et al.* 2003; Butt & Kiehn 2003; Kiehn & Butt 2003; Jankowska *et al.* 2005), and it is already clear that their projections, along with their intrinsic and synaptic properties can differ between and within classes (Kiehn 2006). These classes of interneurons are not necessary for rhythmicity since it is possible to elicit an alternating flexor–extensor rhythm from each side of a split spinal cord (Whelan *et al.* 2000). Nevertheless, CCINs represent attractive targets since they can be identified using electrophysiological and genetic

approaches. An interesting anatomical class of CCINs cross the midline and descend to innervate flexor and extensor motoneurons along with Renshaw cells and Ia inhibitory interneurons (Butt & Kiehn 2003). These descending CCINs are heterogeneous and are composed of both excitatory and inhibitory cells (Restrepo *et al.* 2009*b*). It would be interesting to label these circuits since they represent a tractable target for examining pattern generation. One possibility is the use of transneuronal markers to label these classes of CCINs (Standish *et al.* 1995). In the past, this has been accomplished by injecting the trans-neuronal pseudorabies virus tracer into muscles (Lanuza *et al.* 2004). After 1 day, the marker is expressed in premotoneuronal neurons, and since it is packaged with EGFP, the neurons can be identified. However, these methods suffer from several problems. Since the virus tends to propagate to neurons where there are more synaptic projections, it is difficult to discern weak monosynaptic from strong indirect connections (Luo *et al.* 2008). Another approach is to identify classes of neurons using approaches discussed earlier and ensure that they express Cre recombinase. Retrograde virus, such as pseudorabies virus, can then be manufactured so that they will start to replicate only when they encounter Cre expressed in the targeted class of interneurons (DeFalco *et al.* 2001). Once the replication stop is excised by Cre, PRV is free to infect other cells that project onto the targeted class of interneurons. Another technique is to use monosynaptic trans-synaptic labelling (Wickersham *et al.* 2007). Rabies is expressed in cells that one wishes to target, and a special construct is developed which when combined with the rabies virus allows it to trans-synaptically infect the neighbouring presynaptic cell (Wickersham *et al.* 2007). However, since the presynaptic cell lacks rabies the reaction stops there and cannot go any further.

The ability to dissect spinal cord circuits is difficult owing to the heterogenous nature of the spinal cord. In this regard, the development of 'brainbow' mice offers an attractive technique to identify neurons (Livet *et al.* 2007). Brainbow mice essentially mix multiple XFPs together in such a way that over 90 different hues can be identified in neurons. What this means is that single neurons and their processes can be differentiated. Potentially this approach can be used to target individual classes of cells within the spinal cord (Wilson *et al.* 2007). The attractive feature is that the colours are maintained throughout the neurons soma, dendrites and axons. Therefore, it is possible to identify multiple presynaptic inputs onto a cell and determine whether they come from different neurons or from the same cell. Presumably, use of brainbow mice should also make paired intracellular recording easier since one can trace the processes of the presynaptic cell onto the target cell since the colour will remain the same. Currently, the technique is still under development. It relies on the expression of Thy1, which does not label all classes of neurons. Furthermore, synaptic connectivity cannot be unambiguously resolved (Luo *et al.* 2008). That said, this will be a useful technique for tracing projections to and from candidate CPG interneurons within the

spinal cord. As I mentioned at the beginning, the use of these techniques is best suited to examining defined circuits rather than a distributed network. Circuits that would be especially amenable would be CIN or Renshaw cell circuits.

While powerful, these anatomical mapping strategies represent a static representation of network connectivity. Ultimately, functional connectivity and how this alters during actual network activity need to be measured. How can this be accomplished? One possibility is intracellular recordings from pairs of neurons (Jonas *et al.* 1998; Hinckley & Ziskind-Conhaim 2006). This allows the experimenter to directly examine connectivity between the two putative elements in the circuits. However, this approach is hard and the reality is that the number of sampled pairs will be unacceptably low. Ideally, it would be useful to examine connectivity between an identified neuron and the multiple neurons it connects to. In principle, this can be accomplished by recording intracellularly from the first neuron, eliciting a spike, and then recording a spike-triggered average from multiple neurons using voltage-sensitive dye imaging. The advantages of voltage-sensitive dye recordings are that subthreshold depolarizations can be detected and the temporal resolution is excellent (O'Donovan *et al.* 2008; Homma *et al.* 2009). Unfortunately, the spatial resolution of this approach is low since a photodiode array is used to sum the faint changes in fluorescence produced. Another option is to use calcium imaging approaches. Since there are a large number of high-threshold Ca^{2+} channels in cells, Ca^{2+} indicators that are bulk loaded provide a measure of the degree of extracellular spiking in a population of cells (O'Donovan *et al.* 1993, 1994). Since stimulation of a single cell rarely evokes spiking in target neurons, activation of a class of interneurons would need to be performed to detect connectivity using Ca^{2+} imaging. It is possible to do this using either glutamate uncaging (Callaway & Katz 1993) or light-activated channels, such as channel 2 rhodopsin (Nagel *et al.* 2003). The response of the target interneurons could then be measured by loading these cells with a calcium indicator, such as Fura2-AM (Zhang *et al.* 2007).

A key advantage of using light over other approaches (e.g. allatostain) is that the laser can be pulsed at different frequencies, therefore, activation of classes of cells can be graded. For example, expression of channel rhodopsin 2 (ChR2) in classes of interneurons allows the investigator to activate interneurons that incorporate ChR2 simply by pulsing a blue laser onto the area of interest (Zhang *et al.* 2006). ChR2 is a microbial light-activated channel that conducts cationic currents with fast kinetics similar to AMPA conductances (Nagel *et al.* 2003). On the other hand, another opsin, halorhodopsin can be expressed that rapidly hyperpolarizes the neurons it is inserted into when yellow light illuminates the population. Halorhodopsin works by activating a chloride pump that rapidly increases the intracellular chloride concentration (Zhao *et al.* 2008). Since halorhodopsin is activated by pulses of yellow laser light, it is possible to optically activate and inactivate populations of interneurons by using yellow and blue laser

pulses, making the task of sufficiency and necessity tests tractable. In fact, pure optical stimulation and recording experiments can be constructed. These types of strategies have been used to dissect out cortical microcircuits that produce oscillatory activity (Sohal et al. 2009), which points to the potential of optogenetics as a tool for understanding the CPG function. One of the drawbacks of using this approach in the spinal cord is that the neurons comprising the CPG are distributed rostrocaudally across multiple segments. This limits the utility of this approach since it may not be possible to completely illuminate all segments of the cord equally. However, it may be ideal for examining connectivity in pattern-generating circuits that are located within a segment. On the other hand, pharmacological approaches, such as the use of allatostatin (Lechner et al. 2002; Gosgnach et al. 2006; Zhang et al. 2008; Goulding 2009), are of greater use when examining functionality within a distributed network since bath application affects all the tissue. Use of light versus pharmacological approaches needs to be carefully considered depending on the requirement for fast (ms) activation and inactivation or the need for widespread but slower (min) inactivation.

7. CONCLUSIONS

There is little doubt that genetic approaches have provided a plethora of new tools that allow investigators to dissect genetically spinal cord networks. These tools have led to interesting applications of technologies that allow identification and reversible activation of classes of interneurons. In principle, we have at our disposal tools that allow us to test whether a class of interneurons is necessary for rhythmogenesis and furthermore whether activation of that class is sufficient to produce rhythmic drive to the pattern-generating layer and onto motoneurons. Nearly 100 years following the seminal work of Graham-Brown, we are finally getting a peek inside the black box that encompasses the neural network that produces locomotion. Not surprisingly, this work is raising more questions than it is answering. It is unlikely that we will discover a single 'magic bullet' class of interneurons that generates the locomotor rhythm. On the other hand, a comparatively easier task would be to examine connectivity within the pattern-generating layer. For example, selectively inactivating CCINs that coordinate left–right and flexor–extensor locomotion is one example.

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. We have now entered a phase where the field as a whole needs to consider how to interpret this important data. As a field, we need to clarify what are the most tractable targets using genetic and other techniques.

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