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Strategies for delineating spinal locomotor rhythm-generating networks and the possible role of Hb9 interneurones in rhythmogenesis

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Abstract

Despite significant advances in our understanding of pattern generation in invertebrates and lower vertebrates, there have been barriers to the application of the principles learned to the definition of networks underlying mammalian locomotion. Major difficulties have arisen in identifying spinal interneurones in preparations which allow study of neuronal intrinsic properties and the role of identified interneurones in locomotor networks. Recent genetic technologies in which selective expression of fluorescent proteins in specific populations of mouse spinal neurones have provided new avenues of investigation. In this review, we focus on the generation of locomotor rhythm and outline criteria that rhythm-generating neurones might be expected to fulfill. We then examine the extent to which a recently identified population of spinal interneurones, Hb9 interneurones, fulfill these criteria. Finally, we suggest that Hb9 interneurones could be involved in an asymmetric model of locomotor rhythmogenesis through projections of electrotonically coupled rhythm-generating modules to flexor pattern formation half-centres. The principles learned from studying this population of interneurones have led to strategies to systematically evaluate neurones that may be involved in locomotor rhythmogenesis.

Keywords

Post-inhibitory rebound; Locomotion; Conditional bursting; Central pattern generator; Electrotonic coupling

1. Prologue

In 1985, a Wenner-Gren Symposium entitled “Neurobiology of Vertebrate Locomotion” was held. Today, we can appreciate some of the tremendous advances that have taken place in spinal cord locomotor physiology in the two decades since that meeting. It is useful to look back to the chapter written by Peter Getting for the 1985 Symposium (Getting, 1986) and ask ourselves where our successes and failures have been in delineating spinal rhythmic networks involved in the production of locomotion.

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Getting described the following steps as a strategy for studying rhythmic motor behaviour in "small" systems:

1. Describe the behaviour
2. Characterise the motor pattern
3. Identify the motoneurones and interneurones participating in the behaviour
4. Localise neurones involved in the *generation* of the pattern (in today's terms, he would be referring to the generation of the rhythm)
5. Map the synapticconnectivity between the relevant neurones
6. Characterise the cellular properties
7. Manipulate the network, synaptic, or cellular properties to determine the specific role of these factors in the behaviour
8. Combine this information to reconstruct the pattern generator, motor output, and behaviour

Getting stated that in this "top–down" approach, each step followed "as a natural consequence of the preceding steps." He pointed out that there are significant roadblocks in the application of this strategy to the understanding of mammalian locomotion. In 1985, these roadblocks were particularly evident at steps 3 and 4. Therefore, he suggested that an alternative "bottoms–up" approach would be to "search for the building block mechanisms and to study how these might be assembled into a pattern generator" (Getting, 1986).

In this review, we will illustrate a combined approach by focussing on the identification of possible rhythm-generating neurones (step 4) and their cellular properties (step 6). We will then suggest a possible framework (step 8) which may account for the generation of limbed locomotion.

2. Introduction

In the early 20th century, Graham Brown demonstrated that the mammalian spinal cord contains the neuronal circuitry necessary to generate locomotor activity in the absence of both descending and afferent input (Brown, 1911, 1914). Since that time it has become evident that the protean functional organisation of spinal networks provides the flexibility to produce a variety of patterns and speeds of rhythmic output (Grillner, 1981). Nevertheless, there have been significant challenges to progress beyond Getting's second step. In recent years, progress has been made in the identification of neurones involved in some aspects of locomotion (step 3), such as right–left coordination (Butt and Kiehn, 2003; Lanuza et al., 2004; Nakayama et al., 2002; Zhong et al., 2006). However, neither the precise connectivity of locomotor-related spinal interneurones nor their intrinsic properties have been defined. That is, despite the demonstration that many ventral interneurones are rhythmically active during locomotor activity in the cat (Angel et al., 2005; Baev et al., 1979; Gossard et al., 1994; Huang et al., 2000; Jankowska et al., 1967a; Matsuyama et al., 2004; Shefchyk et al., 1990) and rodent (Butt and Kiehn, 2003; Butt et al., 2005; Kiehn et al., 1996; Tresch and Kiehn, 1999; Zhong et al., 2006) little is known about the connectivity of these neurones

(step 5) or their intrinsic properties (step 6), and none have been shown to be involved in the generation of the rhythm (step 4).

It is important to recognise that neuronal connectivity alone is not sufficient to define a rhythm-generating network; the intrinsic properties of the neurones that comprise the network must be understood (Getting, 1989; Johnson et al., 2005; Marder and Goaillard, 2006). The definitions of the neuronal circuitry and corresponding neuronal properties that produce locomotion have, as Getting pointed out, remained the stumbling blocks in understanding mammalian locomotion. However, since Getting's 1986 paper (Getting, 1986), new techniques combined with increasing knowledge of neuronal differentiation during development have initiated a novel classification scheme for spinal interneurones (step 3) and now enable their study both at the single cell and network level (steps 4–7). In this review, we will ask whether interneurones can be identified that provide the timing (or "clock") necessary for locomotor rhythm generation. To address this issue, we will first examine recent approaches used to classify and identify spinal interneurones.

3. Genetic techniques for the identification of mammalian spinal interneurones

Neuroscientists have recognised the need to develop an unambiguous identification scheme for spinal interneurones so that specific neurones can be identified and studied with respect to their role(s) in the production of locomotion (see (Kiehn, 2006)). Although classical electrophysiological techniques can be used in the cat to classify interneurones (Bannatyne et al., 2003; Jankowska, 1992), these techniques have not provided data regarding the intrinsic properties of these neurones, or their roles in locomotor activity. A recent approach is based on the fact that neurones within the ventral half of the developing neural tube arise from five identified progenitor domains, four of which give rise to different populations of ventral horn interneurones (Briscoe et al., 2000; Jessell, 2000; Lee and Pfaff, 2001; Moran-Rivard et al., 2001; Pierani et al., 1999). Each of these classes of interneurone (V0, V1, V2 and V3) can be defined based upon the selective expression of transcription factors in that population. The use of molecular biological approaches to alter the expression or activity of these specific classes of interneurones has allowed for the identification and study of populations of neurones derived from these domains (Gosgnach et al., 2006; Lanuza et al., 2004). Furthermore, by using fluorescent proteins which are driven by the promoters for these transcription factors, it is possible to study and define these populations anatomically (Alvarez et al., 2005) or physiologically (Hinckley et al., 2005; Wilson et al., 2005) in the post-natal animal. Importantly, this has led to the ability to study the intrinsic properties of identified populations of neurones (Wilson et al., 2005).

One such population we have identified is the spinal Hb9 interneurone population. The homeodomain protein Hb9 is an evolutionarily conserved transcription factor, expressed in somatic motoneurones and interneurones of *Drosophila* (Odden et al., 2002), in postmitotic chick motoneurones (Tanabe et al., 1998) and in mouse progenitor and postmitotic neurones (Arber et al., 1999). By using transgenic technology in mice, GFP expression can be driven by a promoter for the Hb9 gene and subsequently neurones that express Hb9 will, for the

most part, express GFP (Wichterle et al., 2002). In these otherwise phenotypically normal animals, adult neurones expressing Hb9 can be identified and defined based on expression of GFP. Interestingly, we have found that, in addition to GFP expression in motoneurones, there is a population of small, clustered glutamatergic GFP positive interneurones located in medial lamina VIII (and ventral lamina X) bordering on the ventral commissure (Wilson et al., 2005 Fig. 1A, B; see also Hinckley et al., 2005). By crossing Hb9::GFP mice with $Hb9^{nlslacZ/+}$ knock-in mice, we demonstrated that the GFP positive neurones in medial lamina VIII were indeed Hb9 positive. We therefore refer to these neurones as Hb9 interneurones and will discuss these further below.

4. Criteria for rhythmogenic neurones in locomotion

In order to determine that a class of interneurones is necessary for rhythm generation, it is essential to demonstrate that elimination of that population disrupts rhythm generation. Furthermore, the class can be said to be sufficient for rhythm generation if selective activation of those cells initiates the rhythm. Although some neuronal classes have been shown to affect the speed of locomotion (e.g. Gosgnach et al., 2006; Kjaerulff and Kiehn, 1997), to date no class of interneurone in the mammalian spinal cord has been shown to be either necessary or sufficient for locomotor rhythmogenesis. In order to proceed with such loss or gain of function experiments, it is first necessary to identify candidate interneuronal populations which may be involved in generating this rhythm. The question then arises: how would we recognise a population that is involved in rhythm generation? By combining principles learned from the study of rhythm-generating interneurones in invertebrates and lower vertebrates as well as from modeling studies with findings from the study of mammalian spinal cord locomotor networks, it is possible to compile a list of specific criteria which would be expected of rhythm-generating neurones. Specifically, neurones involved in locomotor rhythm generation should have many of the following properties:

- **a.** They are located in thoracolumbar spinal cord (Cazalets et al., 1995; Cowley and Schmidt, 1997; Kjaerulff and Kiehn, 1996; Marcoux and Rossignol, 2000)
- **b.** They are in the ventromedial spinal cord (Kjaerulff and Kiehn, 1996);
- **c.** They receive glutamatergic reticulospinal (Douglas et al., 1993; Noga et al., 2003; Ohta and Grillner, 1989) and descending serotonergic input (Liu and Jordan, 2005; MacLean et al., 1998; Ribotta et al., 2000);
- **d.** They receive primary afferent (group I) input, evidenced by the ability of primary afferent stimulation to “reset” the rhythm (Conway et al., 1987);
- **e.** Their transmitter phenotype is glutamatergic (Lundberg, 1981);
- **f.** There is mutual excitation or recurrent (self) excitation of these neurones (Roberts and Tunstall, 1990; Rowat and Selverston, 1997);
- **g.** They are not last order premotor interneurones (Burke et al., 2001) (see McCrea and Rybak, 2008 and “modeling” below; but see Kiehn, 2006 for further discussion);

h. Their intrinsic properties will include mechanisms for rhythm generation such as post-inhibitory rebound (PIR) (Perkel and Mulloney, 1974; Tegner et al., 1997), plateau potentials (Russell and Hartline, 1978), spike frequency adaptation (el Manira et al., 1994) and/or conditional bursting properties (Getting, 1986); and

i. They must be rhythmically active in response to drugs that induce locomotion (Jankowska et al., 1967a; Jiang et al., 1999; Kudo and Yamada, 1987), in response to a rise in excitability (Bracci et al., 1998; Taccola and Nistri, 2004) and during locomotion itself.

Whether the neurones involved in the generation of the rhythm are themselves intrinsic (conditional) bursting neurones or whether the rhythm results from the connectivity of interneurones is currently not known. Either mechanism (or a combination of the two) may lead to stable, alternating rhythm generation see (Marder and Bucher, 2001; Getting, 1989; Perkel, 1976). It is interesting to note that, based on research in invertebrates, Getting (1986) predicted that, to generate an intermittent bursting pattern as seen during mammalian locomotion, the neurones involved in rhythm generation would likely be conditional bursters.

A useful approach to define mammalian spinal cord locomotor rhythm-generating networks would therefore be to identify interneuronal populations, determine the extent to which they fulfill the above criteria and then to study their intrinsic and network properties. This approach lies in between Getting's top–down and bottoms–up strategies. Once a candidate population is identified, the questions of necessity and/or sufficiency for rhythm generation can be addressed. Prior to describing Hb9 interneurones and the extent to which they fulfill these criteria, we will briefly discuss the importance of studying neuronal intrinsic properties in rhythm-generating networks.

5. Neuronal intrinsic properties and rhythm generation

It is clear that knowledge of synaptic connectivity of motor circuits in the absence of knowledge of the neuronal properties will be insufficient to understand motor pattern generation (Getting, 1989; Grillner, 1981; Harris-Warrick, 1993; Hooper and DiCaprio, 2004; Katz and Frost, 1996; Marder and Bucher, 2001). From studies in invertebrates, for example, it is known that the intrinsic properties of network neurones function to both generate the rhythm and to pattern the output (Getting, 1989). This has been demonstrated in the 14-neurone pyloric network of the crustacean stomatogastric ganglion, in which, for example, the transient voltage-activated potassium channel I_A is expressed to variable degrees in the different neurones and plays an important role in the function of the individual neurones as well as in network function (Tierney and Harris-Warrick, 1992). When this conductance is blocked pharmacologically, isolated neurones fire earlier following their hyperpolarised phases and fire with increased frequency. In addition, blocking I_A in the intact network leads to a decrease in cycle period and an alteration in the phasing of the follower cells. Furthermore, application of the neuromodulator dopamine to the intact network leads to an increase in cycle frequency through variable mechanisms in different cell types (Harris-Warrick et al., 1998). In the pyloric constrictor (PY) cells, for example, dopamine decreases the delay to onset of firing and increases firing frequency, effects

mediated by a reduction in I_A (Harris-Warrick et al., 1995a). Taken together, these studies have beautifully demonstrated the dynamic interaction of intrinsic properties and network activity, and the harmonisation of the control of these properties and the network by neuromodulators.

The intrinsic properties of pattern generating neurones must support the generation of excitatory, transitional and inhibitory phases of the network rhythm. Combinations of several different properties, such as plateau potentials, spike frequency adaptation, endogenous bursting and/or PIR, are necessary for rhythm and pattern generation (Marder and Bucher, 2001). Plateau potentials ensure continued action potential firing in the absence of sustained synaptic input (Conway et al., 1988; Russell and Hartline, 1978). Spike frequency adaptation contributes to termination of a burst (el Manira et al., 1994; Sun and Dale, 1998). Rhythm may be generated by either endogenously bursting neurones or by reciprocally inhibitory neurones (Getting, 1989; Satterlie, 1985). PIR is particularly important in the generation of the rhythm both in endogenous bursters (to ensure a rapid onset to the burst) and in reciprocally inhibiting networks (even if these are not endogenous bursters, two reciprocally inhibiting neurones can generate rhythmic output if they both have PIR (Mulloney et al., 1981; Perkel and Mulloney, 1974)). PIR is also crucial for the timing of action potential burst onset and the pattern of firing in many rhythmic networks (Arshavsky et al., 1998; Eisen and Marder, 1984; Harris-Warrick et al., 1995b; Hartline and Gassie, 1979; Roberts et al., 1995) and may also contribute to the stability of the rhythm (Tegner et al., 1997). These data have led to the hypothesis that PIR is a desired property in rhythm-generating networks (Hooper and DiCaprio, 2004).

In summary, numerous studies indicate that the frequency and phasing of the rhythm depend on the interplay of intrinsic properties of the neurones and the strength and time-course of synaptic interactions between them.

6. Role of electrotonic coupling in rhythm generation

Electrotonic coupling between interneurones may facilitate network rhythm generation across diverse systems. For example, in Tritonia, some interneurones involved in swimming activity are electrotonically coupled (Getting et al., 1980). In the stomatogastric ganglion (STG) of the lobster, gap junctions connect three different interneuronal populations involved in rhythm generation (Eisen and Marder, 1982). Electrotonic coupling of neurones of the pre-Bötzinger complex and hypoglossal nucleus may generate or modulate the respiratory rhythm in mammals (Rekling et al., 2000). Thus electrotonic coupling can be present both within a population of rhythm-generating neurones (homogeneous coupling) and between populations of neurones involved in rhythm generation (heterogeneous coupling).

Electrotonic coupling has been shown to facilitate synchronous spiking activity across pairs and populations of different types of neurones (see Connors and Long, 2004 for review). The firing patterns and degree of synchrony of coupled cells depend on the coupling conductance, as demonstrated by modeling studies of the crustacean stomatogastric ganglion (Sharp et al., 1992) and the mammalian inferior olive (Ozden et al., 2004). These studies

show that neurones may fire independently in a weakly coupled system. As coupling strength increases, neurones may become phase-locked (i.e. in or out of phase depending on coupling parameters and intrinsic properties of the neurones). Interestingly, at high coupling conductances oscillations completely cease (Ozden et al., 2004). In addition to synchronous firing, electrotonic coupling can also promote and/or stabilise bursting activity (Getting and Willows, 1974; Skinner et al., 1999) and regulate the interburst interval (Sharp et al., 1992). Furthermore, although individual neurones need not be endogenous pacemakers to elicit a synchronous rhythm in a coupled network (Manor et al., 1997; Sherman and Rinzel, 1992; Smolen et al., 1993), intrinsic pacemaker properties of individual neurones within an electrotonically coupled network can facilitate bursting activity (Sherman and Rinzel, 1992). In summary, electrotonic coupling is a common feature in rhythm-generating networks and can endow these networks with stable bursting properties that may be subject to neuromodulation.

7. A candidate population of rhythmogenic neurones: Hb9 interneurones

We have demonstrated that Hb9 interneurones satisfy many of the criteria listed above for neurones involved in locomotor rhythm generation (Wilson et al., 2005): a thoracolumbar (Fig. 1A) and ventromedial (Fig. 1B) location, serotonergic (Fig. 1C) and primary afferent (Fig. 1D) input and glutamatergic phenotype (Fig. 1E). Although we presented anatomical evidence of possible mutual or self-excitation (Fig. 1F), neither Hinckley and Ziskind-Conhaim (2006) nor we (Wilson et al., 2007) have found supportive electrophysiological evidence of this. In addition, we have been unable to demonstrate terminals on motoneurones (Fig. 1G) rendering it unlikely that these neurones are last-order. However, some evidence has been presented that GFP positive neurones in this region send processes to the motor pools (Hinckley et al., 2005). This remains an unresolved question. Thus, Hb9 interneurones fulfill at least 6 of 9 criteria and therefore warranted further investigation.

As Getting suggested, it is possible that the rhythm of mammalian locomotion is produced by activity in conditionally bursting interneurones (Getting, 1986). We therefore explored whether Hb9 interneurones have intrinsic properties to support endogenous bursting. Electrophysiological studies of Hb9 interneurones in spinal cord slices from “motor functionally mature” mice (i.e. of a developmental stage at which they could weight bear and walk; see Jiang et al., 1999) reveal a post-inhibitory rebound (PIR) potential mediated by T-type calcium channels (Fig. 1H, Wilson et al., 2005). When Hb9 interneurones were synaptically isolated in spinal cord slice experiments by adding TTX to the perfusate and the neuro-transmitters that induce fictive locomotion in these older animals (serotonin, dopamine and NMDA) were added (Jiang et al., 1999), Hb9 interneurones became rhythmically active. This indicated that they did have an endogenous rhythm-generating capacity (Fig. 1H, Wilson et al., 2005).

Further evidence implicating Hb9 interneurones in locomotor networks comes from their expression of the activity-dependent protein, Fos, following a locomotor task in the adult mouse (Wilson et al., 2005, Fig. 1I). In addition, GFP positive neurones in this area in the hemisected spinal cord are rhythmically active during drug-induced rhythmic activity in the newborn mouse (Hinckley et al., 2005, Fig. 1I). Furthermore, Hb9 interneurones exhibit

rhythmic and stable bursts of action potentials in response to a generalised increase in excitability (Wilson et al., 2007). Thus, Hb9 interneurones satisfy at least parts of all 9 criteria described above for locomotor rhythmogenic neurones (Fig. 1).

Two additional findings regarding the conditional oscillations were observed: (a) in some Hb9 interneurones, the oscillations were voltage-independent, leading us to suggest that these neurones were electrotonically coupled to other neurones with oscillating membrane potentials (Wilson et al., 2005); and (b) in other Hb9 interneurones, the oscillation period decreased with depolarisation. We will return to the latter point below (Regulation of locomotor speed: V1 and Hb9 interneurones). With respect to the former point, we have recently found that Hb9 interneurones are not electronically coupled to each other, but to other, non-Hb9 interneurones (Wilson et al., 2007) which had previously been shown to have spikelets indicative of electrotonic coupling (Wilson et al., 2005). This is in contrast to another recent study which suggested that putative Hb9 interneurones are coupled with each other (Hinckley and Ziskind-Conhaim, 2006).

In summary, Hb9 interneurones fulfill the majority of the criteria necessary for locomotor rhythm-generating neurones and are possibly electrotonically coupled to neurones with intrinsic properties that may differ from those of Hb9 interneurones. Indeed, electrotonic coupling between heterogeneous populations of rhythm-generating neurones imparts a network with the capacity to produce a flexible, yet stable, rhythm (Soto-Trevino et al., 2005). Future experiments on Hb9 interneurones will address the necessity and sufficiency of Hb9 interneurones in the production of locomotion.

8. Modeling locomotor networks

Although we are not yet at the point where we can “reconstruct” the network (Getting’s step 8), we can examine whether and how neurones like Hb9 interneurones can be incorporated into models of spinal locomotor networks. To do this, we will first briefly discuss some current locomotion modeling issues.

8.1. Pattern formation and rhythm-generating modules

In the absence of detailed knowledge about the connectivity of neurones involved in producing locomotor output, it is difficult to predict exactly what the network “looks like” (eighth and ultimate step of Getting’s principles; Getting, 1986). This becomes even more daunting in the absence of detailed knowledge of the electrophysiological properties of the neurones within the network. In fact, in 1981 Grillner wrote: “Although it is meaningful to try out ideas about how a conceived network could operate, it is obvious that at present no model can qualify for more than an important stimulus for the thought process” (Grillner, 1981). Although one can readily make a model(s) that will emulate observed behaviour, caution must be exercised in the interpretation of the model. For example, Prinz and colleagues (Prinz et al., 2004) modeled the 3-neurone rhythm-generating circuit of the STG and tested 20,000,000 different sets of neuronal parameters. They found that 500,000 of these sets produced appropriate biological output. They concluded that great variability in properties may exist from animal to animal, yet these properties were sufficiently constrained to ensure that the network output remained remarkably stereotypical. Two

principles relevant to the current review emerge from this study: (a) variability in neuronal connectivity and intrinsic properties may exist between animals, despite stereotypical behaviour; and (b) the prediction of a behaviour from a model does not imply that the model accurately reflects the biology. In order to determine the accuracy of a model, it is not only necessary that the model emulates the behaviour, but it is also necessary to demonstrate that the model responds appropriately to novel inputs, and further that new physiological experiments are suggested and their outcomes accurately predicted (Kristan and Katz, 2006). Despite this, there is no doubt that we have learned much from modeling studies of vertebrate locomotion to date (Grillner and Wallen, 2002; Tunstall et al., 2002). There is little doubt that as we develop more constraints on the possible models of mammalian locomotion, the utility of these models will improve.

Notwithstanding, it is evident that the results from electrophysiological studies of mammalian locomotion have led to some general principles which have enabled the formation of general conceptual models. In the early 20th century, Graham Brown proposed that the spinal networks were organised in “half-centres.” By this, he meant in contemporary terms that interneuronal modules for coordinating flexor and extensor activity reciprocally inhibit each other. This model was re-visited by Lundberg (1981), who illustrated that the organisation of reflex pathways (studied by stimulating flexor reflex afferents) was consistent with a half-centre organisation for locomotion. Lundberg suggested that the central organisation was such that flexor and extensor activities throughout the limb were produced by two half-centres and that proprioceptive afferent input then “sculpted” the activity in individual muscle groups. Following this, however, it became clear that the individual patterns of activity of specific muscle groups could be produced during fictive locomotion in the absence of afferent input (e.g. Pearson and Rossignol, 1991). Therefore, such patterns were centrally controlled and could not be explained by having single half-centres for flexion and extension within a limb.

In light of these findings which demonstrated that there could not be single half-centres for each limb, Grillner proposed a unit burst generator model. This model consisted of multiple interconnected modules for flexion and extension for the different joints in a limb (Edgerton et al., 1976). Although a more distributed system, each module can indeed be considered to be as a half-centre, as flexor and extensor activities across each joint are indeed reciprocally activated. We therefore proposed that the term “half-centre” should not be used in the Graham Brown/Lundberg sense, but rather the definition should be loosened and used in a more “eclectic” fashion so as not to “straight-jacket” the thinking of spinal locomotor networks (Hultborn et al., 1995). In this review, the term “half-centre” will therefore be used to indicate reciprocal inhibition between flexor and extensor modules governing movement around a single joint (Fig. 2). The modules that govern activity across different joints would be interconnected in some fashion, as proposed by Grillner (Edgerton et al., 1976).

A major question that remains is whether these half-centre modules that ensure reciprocal activity of flexors and extensors (“pattern” of activity) include the neurones which generate the rhythm. In lower vertebrates which locomote by undulation, it has become clear that the neurones responsible for rhythm and pattern are one and the same (Lansner et al., 1998; Roberts et al., 1998). These animals do not have the complexity of multiple flexor and

extensor muscles within a limb; that is, limbed vertebrates must control both intralimb and interlimb coordination. How did the networks involved in this increased complexity evolve? For example, did the circuits simply evolve in a “lateral” fashion, being reproduced on each side of the spinal cord (i.e. use similar circuitry for flexion-extension as undulating vertebrates use for left–right coordination)? Did new levels of the circuit develop to regulate timing, or could the rhythm be produced within each module?

Several studies present evidence that the locomotor network is “two-layered,” in that the rhythm is produced by neurones independent of those responsible for the pattern, (see Fig. 2; Lafreniere-Roula and McCrea, 2005; Rybak et al., 2006a,b and McCrea and Rybak, 2008; see Kiehn, 2006 for additional discussion on this point). Although there is much evidence that the last-order, pattern-forming circuitry is organised in half-centre modules (see above), there is little evidence that the rhythm-generating layer follows suit. While McCrea and colleagues present some compelling evidence in support of a two-layer model, their evidence that the rhythm-generating layer is organised as half-centres is much less convincing. These investigators rely on spontaneous (Lafreniere-Roula and McCrea, 2005; Rybak et al., 2006a) or evoked (Rybak et al., 2006b) “deletions” of activity in single motor nerves that either are (“resetting” deletions) or are not (“non-resetting” deletions) associated with disruptions of rhythm. They reason that since these non-resetting deletions can be seen in either flexor or extensor muscle nerves, the rhythm-generating layer must excite both the flexor and extensor pattern formation half-centres. There are two weaknesses to this argument: (a) in the absence of recordings from muscle nerves across all joints in the contralateral and front limbs, it cannot be ruled out that the maintenance of rhythm is maintained by input from rhythm-generating neurones in those limbs; and (b) in the absence of detailed knowledge of the intrinsic properties of the neurones involved, the cause of the deletions cannot be determined — for example, they could be produced by plateau potentials in inhibitory interneurones in the pattern formation layer. Furthermore, it must be stressed that insights into locomotor network organisation come from motoneuronal recordings. Since the rhythm-generating layer is not likely to be monosynaptically connected to motoneurones (in the two-layer hypothesis; see above, Fig. 2), activity in rhythm-generating neurones can only be inferred, and failures due to disynaptic connectivity must be considered. Given the above considerations, it is essential that we consider a variety of possibilities of organisation of the rhythm-generating layer. For example, the rhythm-generating layer need not necessarily have half-centre flexor–extensor organisation (i.e. symmetrical rhythm generator).

8.2. Possibility of asymmetry in rhythm-generating networks

Several lines of experimental evidence from different species of limbed animals indicate that a rhythm-generating layer may indeed be asymmetric. In the cockroach locomotor system (a single-layered rhythmogenic network), a population of oscillating interneurones are responsible for alternating flexor–extensor rhythmic drive. Based upon the experimental evidence that rhythmic bursting activity in flexor motoneurones can continue in the absence of extensor bursts (Pearson, 1972; Pearson and Iles, 1970), it was proposed (Fourtner, 1976; Pearson and Iles, 1970) that these neurones preferentially excite flexor motoneurones. To entrain the extensor motoneurones, the rhythm-generating kernel projects to an inhibitory

interneurone that innervates the extensor motoneurones. Thus, the rhythm generator can determine and reset the cycle time of the intralimb pattern (Fourtner, 1976) by monosynaptically driving the flexors and disynaptically inhibiting the extensors (cf. Fig. 3, below).

It has been more difficult to establish whether or not the mammalian locomotor rhythm-generating network is symmetric. The difficulty arises from our inability to define the neurones involved in rhythmogenesis, and from the fact that our models rely on data obtained from motoneuronal recordings, which are likely disynaptically "removed" from the neurones involved in rhythm generation. Evidence has been presented in favour of (Duysens, 1977) and opposed to (Lafreniere-Roula and McCrea, 2005) the concept that this is an asymmetric network. This topic has recently been discussed in an exchange of letters to the editor in the *Journal of Neurophysiology* (Duysens, 2006). The arguments will therefore not be repeated here. Suffice it to say that given experimental limitations, we cannot exclude the possibility that rhythmogenesis is asymmetric.

In summary, the locomotor network can be considered to have a rhythm generation "layer" presynaptic to a last order pattern formation "layer." There is excellent evidence supporting a flexor–extensor half-centre model in the pattern formation layer, but the organisation of the rhythm-generating layer remains enigmatic (Fig. 2).

8.3. Coupled oscillators

As indicated above, there is evidence that flexor and extensor half-centres in the pattern formation layer reciprocally inhibit each other (Fig. 2). The nature of this connectivity in itself would imply that this layer is autonomously capable of generating rhythm should two conditions be met: (a) the excitation of the network is sufficient to produce firing in the excitatory interneurones of the half-centres; and (b) there is a mechanism by which these neurones "fatigue," such as spike frequency adaptation (see Marder and Bucher, 2001). Since it isunlikely that this layer is responsible for locomotor rhythm generation (see above), one must consider, in addition to the coupling of oscillators across many segments or joints, the effects of the coupling of several different types of oscillating circuits — each rhythm-generating layer (bilaterally represented and coupled with each other) and the pattern generating layers in each hemicord (see Cohen et al., 1992). Further studies of the interactions of heterogeneous oscillators distributed across multiple levels will be useful in defining the contribution of each of these potentially oscillating components to locomotor rhythm generation.

9. A proposed asymmetric model of rhythm generation

Given that Hb9 interneurones fulfill many of the criteria necessary for locomotor rhythmogenic neurones (see above), including that they are conditional endogenous bursters (see above, and Wilson et al., 2005), that during rhythmic activity they appear to burst in phase with ventral root activity (Hinckley et al., 2005), that locomotor rhythmogenesis seems to arise in large part from the part of the spinal cord which produces flexor-related bursting activity, L2 (Cazalets et al., 1995; Cowley and Schmidt, 1997; Kjaerulff and Kiehn, 1996; Marcoux and Rossignol, 2000), and that the rhythm generator may not be organised in

a symmetric flexor–extensor half-centre network, we would propose the possibility that the locomotor network may be arranged as illustrated in Fig. 3. In this model, we propose that the rhythm-generating layer is composed of a kernel of heterogeneous, electrotonically coupled neurones that project directly to the flexor half-centre of the pattern formation layer. Alternating gait would then be produced by reciprocal inhibition between the rhythm generators on either side of the spinal cord as illustrated in Fig. 3. In animals which can also gallop, this reciprocal inhibition could be replaced by reciprocal excitatory connections. For clarity in the figure, we have not illustrated details of crossed pathways of the pattern formation layer that nevertheless must exist (see Lundberg, 1981; Jankowska et al., 1967a,b). Note that the two rhythm generators (on either side of the spinal cord) form a right–left "half-centre," which would likely also contribute to rhythm generation (Fig. 3). This simplified model yields many testable questions that can be pursued in future experiments.

10. Regulation of locomotor speed: V1 and Hb9 interneurones

The neurones and networks involved in generating a locomotor rhythm must have the capacity to adjust the cycle period to account for varying speeds of locomotion. Little is known of the mechanisms of speed control in mammals (Kriellaars et al., 1994; Orlovsky and Shik, 1976). Recently, however, studies in newborn mice revealed that the cycle period of locomotor output lengthened when neurones of V1 lineage were silenced (Fig. 4A, Gosgnach et al., 2006). Since V1 neurones are inhibitory interneurones (Alvarez et al., 2005), this study demonstrated that removal of inhibition slows the locomotor speed. This finding is complementary to that found in Hb9 interneurones, in which the frequency of bursting was reduced when the membrane was depolarised (Fig. 4B; Wilson et al., 2005). These two, perhaps counterintuitive, findings could in fact be coherent if one hypothesises that V1 inhibitory neurones project to rhythm-generating Hb9 interneurones (Fig. 4C). In this hypothesis, silencing V1 interneurones would remove the inhibition of Hb9 interneurones, induce depolarisation and thus slow the burst frequency and hence locomotor speed. The demonstration that V1 interneurones inhibit Hb9 interneurones would support this hypothesis and further implicate Hb9 interneurones in locomotor rhythm generation.

11. Closing comments

In this review we have examined Getting's outline in relation to the study of mammalian locomotor networks (Getting, 1986). In addition, we have suggested criteria by which newly defined populations of interneurones can be studied with respect to their potential involvement in locomotor rhythmogenesis. We illustrate that a recently identified population of interneurones, Hb9 interneurones, fulfill many of the criteria and therefore warrant further investigations into whether they are either necessary or sufficient for locomotor rhythm generation. Finally, we have proposed an asymmetric model for rhythm generation in locomotion from which testable hypotheses can be derived. We cannot conclude that Hb9 interneurones are involved in the generation of the locomotor rhythm. Nevertheless, the principles learned from studying this population of interneurones have led to strategies with which we can systematically evaluate neurones that may be involved in locomotor rhythmogenesis.

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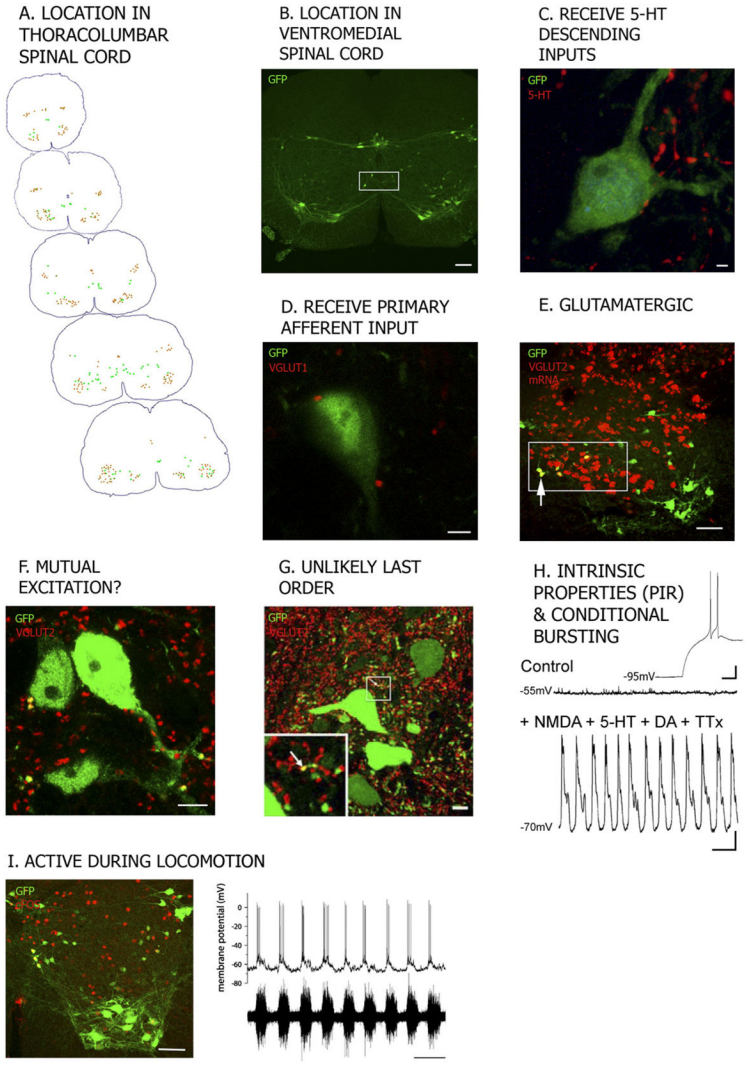

Fig. 1.

Hb9 interneurones fulfill many of the criteria for rhythm-generating interneurones. A combination of anatomical and electrophysiological studies in Hb9::GFP mice defines the properties of Hb9 interneurones and determines their suitability for rhythm generation. (A) Neurolucida mapping studies of transverse spinal cord sections from mid-thoracic to mid-lumbar levels reveal GFP+ interneurones (green) extending from the thoracic to the mid-lumbar spinal cord. GFP+ somatic and autonomic motoneurones are indicated in brown. The clusters of GFP+ interneurones abutting the ventral commissure are not seen below the mid-lumbar level. By crossing this strain with $Hb9^{nlslacZ/+}$ knock-in animals, it was revealed that this population of neurones is Hb9+ (not shown). (B) A representative transverse spinal cord section demonstrates the ventromedial location of Hb9 interneurones (box). (C) An Hb9 interneurone (labeled with anti-GFP, green) is contacted by serotonergic fibres (anti-5-HT, red). (D) VGLUT1 terminals (red) oppose the cell body of an Hb9 interneurone providing evidence that these neurones receive primary afferent input. (E) Fluorescent in situ hybridisation reveals that Hb9 interneurones (green, arrow) contain the mRNA for VGLUT2

(red) thus revealing a glutamatergic transmitter phenotype. (F) Hb9 interneurones (green) are contacted by VLGUT2 positive terminals (red) that are also GFP-positive, raising the possibility that Hb9 interneurones are reciprocally connected. (G) A medium power image indicates the scarcity of VGLUT2+ (red), GFP+ (green) terminals (double positive, yellow, arrow, inset) in lamina IX, making it unlikely that Hb9 interneurones provide significant input to motoneurones. (H) Whole-cell patch clamp recordings from an Hb9 interneurone reveal a prominent post-inhibitory rebound (PIR) potential leading to multiple action potentials when the neurone is released from a hyperpolarising potential (arrow). Hb9 interneurones undergo large oscillations in membrane potential in the presence of NMDA, 5-HT, dopamine and TTX, indicating that they are conditional bursters. Scale bars in inset are 10 mV and 100 ms, and for the oscillations 10 mV and 5 s. (I) Hb9 interneurones (green) are active in locomotor activity as demonstrated by the presence of Fos protein (red) following a locomotor task in the adult. In addition, their activity in the neonatal hemisected spinal cord is in phase with the output of an unidentified ventral root. (Scale bars are 100 μm in panels B, E and I, 10 μm in G, 5 μm in F and D, 2 μm in C, 20 s in panel I. Panels B, C, D, E, F, G and H, and left side of I from Wilson et al. (2005). The right-sided portion of panel I is from Hinckley et al. (2005). With permission.).

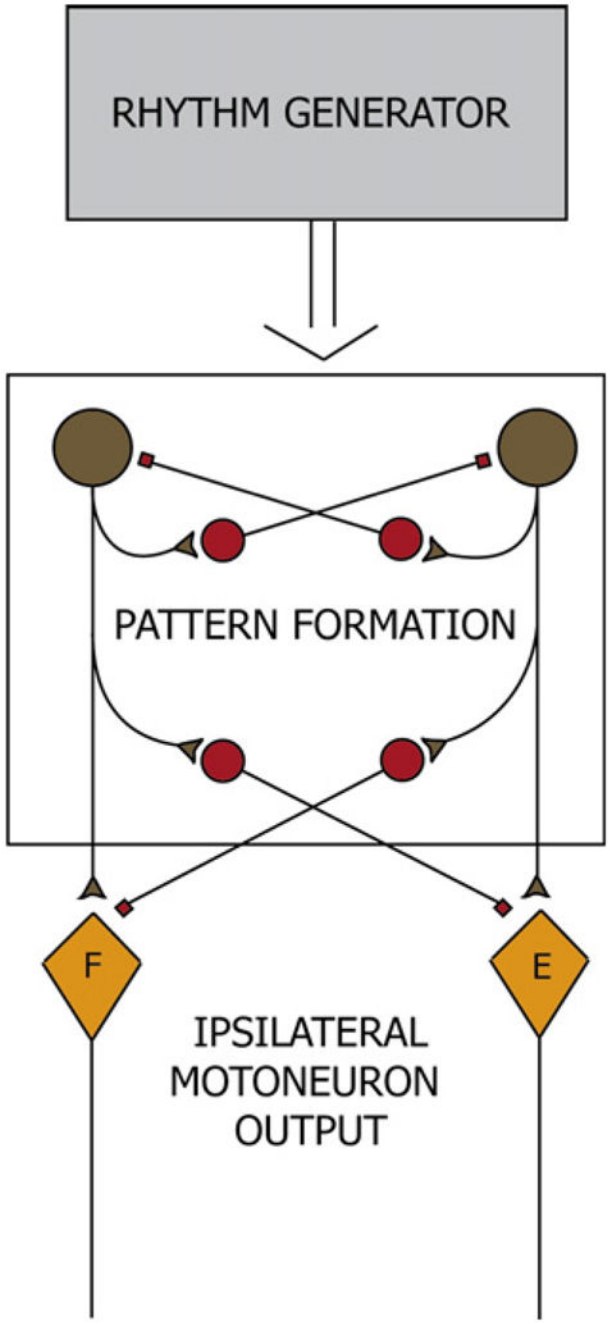

Fig. 2.

A schematic diagram similar to Brown's model illustrated, then modified, by Lundberg (1981). This shows a half-centre model for a pattern formation layer in the mammalian locomotor network. The flexor (F) and extensor (E) motoneurones receive input from last-order excitatory interneurones that are reciprocally innervated by inhibitory interneurones (red). Another population of inhibitory interneurones ensures inhibition of the antagonist motor pools. Although this half-centre model may in itself be capable of generating a rhythm, evidence is presented that the rhythm-generating portion of the network is distinct

and forms another “layer” (see text). Little is known about the rhythm generator, which is thus represented by a grey box.

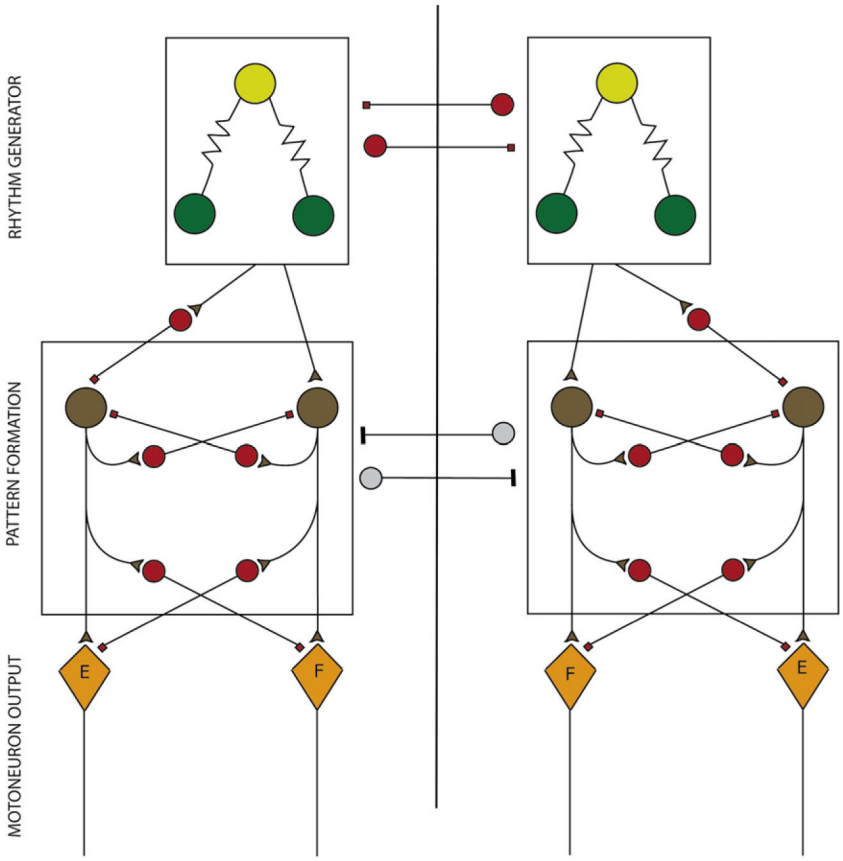

Fig. 3.

A schematic diagram suggesting a possible flexor-dominated rhythm generator for mammalian locomotor output. The rhythm generator consists of a kernel of Hb9 interneurones (dark green) that are electrotonically coupled to non-Hb9 interneurones (light green). This network produces rhythmic output which excites the corresponding last-order flexor-related interneurones of a half-centre. The rhythmic output also leads to disynaptic inhibition of the extensor-related half-centre interneurones. The rhythm generator is reciprocally innervated by the rhythm generator on the contralateral side to ensure interlimb coordination. In the case of alternating gait, these reciprocal connections will be inhibitory (red). In addition, connections between the bilateral pattern formation layers are also indicated (grey), although the details of these are not known (but see Lundberg, 1981; Jankowska et al., 1967a,b).

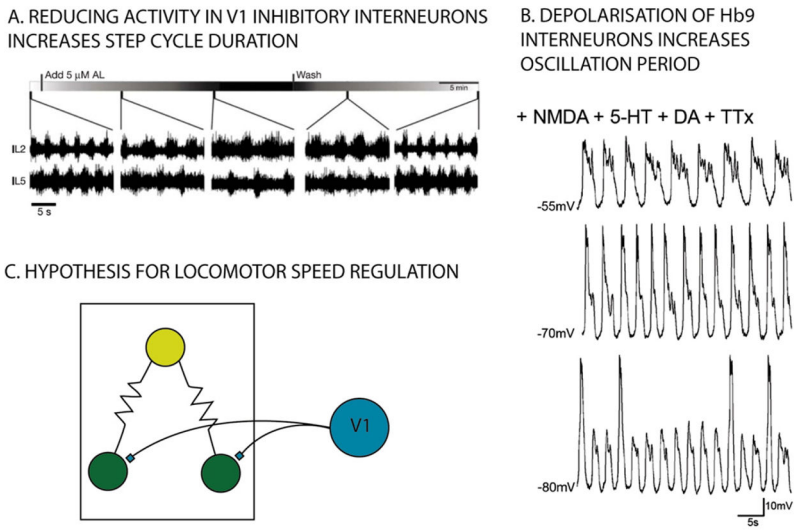

Fig. 4.

Hypothesis for the regulation of locomotor speed: relationship of V1 and Hb9 interneurones. (A) Locomotor speed slows down (step cycle increases in duration) when the activity of inhibitory V1 interneurones is depressed (Gosgnach et al., 2006). In these transgenic mice, V1 interneurones express the receptor for allatostatin, which is coupled to a G-protein coupled inward rectifying potassium (GIRK) channel. When the ligand is present (AL), V1 interneurones hyperpolarise and thus their output is reduced. This leads to a reversible decrease in locomotor speed. That is, removal of V1 interneurone mediated inhibition leads to a slowing of the rhythm. (B) Depolarisation of Hb9 interneurones increases oscillation period. (C) These findings lead to the hypothesis that inhibitory V1 interneurones innervate Hb9 interneurones. When they are removed, Hb9 interneurones depolarise, leading to an increase in their interburst interval and therefore a reduction in locomotor speed.