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# Circuits controlling vertebrate locomotion: moving in a new direction

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## Abstract

Neurobiologists have long sought to understand how circuits in the nervous system are organized and generate the precise neural outputs that underlie particular behaviors. The motor circuits in the spinal cord that control locomotion and are commonly referred to as central pattern generator (CPG) networks, provide an experimentally tractable model system for investigating how moderately complex ensembles of neurons generate select motor behaviors. The advent of novel molecular genetic techniques coupled with recent advances in our knowledge of spinal cord development means that a comprehensive understanding of how the motor circuitry is organized and operates may now be within our grasp.

Motor tasks are key components of the behavioral repertoire of all animals [1–3]. While animals typically exhibit quite varied and complex patterns of motor activity, many of the simpler motor behaviours they display –breathing, chewing, peristalsis, swimming, scratching and walking –are well suited to experimental analysis [4–13]. The analysis of motor behaviors has long been at the centre of efforts to understand how the nervous system is organized and functions, with Sherrington's studies providing important insights into the integrative nature of neural pathways, the reflex arc and the control of reciprocal motor actions by central inhibitory pathways [14]. Sherrington's efforts were based on his recognition that motor neurons as neural effectors for motor actions constitute "the final common pathway". Subsequent studies in the cat, by Eccles, Lundberg, Jankowska and colleagues went a long way toward defining the spinal reflex circuitry, including the properties of the constituent interneurons and their actions on motor neurons 15–18. Whereas Sherrington favoured the idea that complex motor behaviors, including locomotion, were generated by chains of reflex actions 14, Brown countered this idea by providing evidence that intrinsic networks in the spinal cord can generate rhythmic locomotor-like patterns of activity [19]. This observation gave rise to the concept of the central pattern generator (CPG), a neuronal network that is capable of generating an organized pattern of motor activity independently of sensory inputs, which was first described in invertebrates [20]. In the spinal cord, such networks function as local "control and command" centers 11–13, 21 to generate rhythmic axial and limb movements. Descending inputs from the brainstem, basal ganglia and cortex control the selection and shaping of outputs from the locomotor CPG, with further layers of modulation coming from sensory and vestibular pathways that converge on CPG neurons (Figure 1) [22–25].

The vertebrate locomotor CPG comprises a distributed network of interneurons and motor neurons, which upon appropriate stimulation, generates an organized motor rhythm that replicates the patterns of motor activity seen during repetitive locomotor tasks such as walking and swimming (Figure 2). The central organizing feature of the motor circuitry is the grouping

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of motor neurons into discrete operational units, motor pools, each of which innervates a single muscle [14]. The graded recruitment and activation of motor neurons within a pool [26–28] underlies the variable changes in muscle tension that are necessary for smooth muscle movement and postural control. Motor neurons are able to integrate a range of convergent inputs, although it is likely that much of the integration that generates coordinated motor activity in the takes place upstream in the locomotor interneuron network. Fast synaptic and slower modulatory interactions between locomotor interneurons sculpt the patterns of motor neuron activity that coordinate limb and body movements, however, these interactions remain largely undefined.

Past efforts to probe the neuronal networks responsible for generating motor behaviors have typically utilized classical systems neuroscience approaches, such as combinations of neuronal recordings and pharmacological manipulations. Although these studies have provided important insights (see above) they, are limited by difficulties in reproducibly identifying and manipulating the neurons within these networks. In recent years, the convergence between developmental genetics and physiological and behavioral systems approaches has opened up new avenues for molecularly identifying and manipulating specific neuronal cell types. In this review, I will highlight how efforts to understand the genetic regulation of neuronal specification in the embryonic spinal cord are now being merged with systems approaches to study the organization of the spinal locomotor network in vertebrates [29–38]. In particular, the increased sophistication of genetic manipulations in mice and other model vertebrate organisms such as zebrafish [39–41] is facilitating a more precise dissection of the motor circuitry and the contribution that different neuronal cell types make to motor behaviours.

## Lessons from other rhythmic model systems

The cellular mechanisms that underlie the rhythmic motor behaviors produced by CPG networks have been explored in a number of contexts, including insect flight, swimming in the mollusc *Clione*, gut movements in crustaceans, as well as swimming and respiration in vertebrates [3–6,11]. The relative simplicity of these systems, best exemplified by the crustacean stomatogastric nervous system (STG), have facilitated efforts to define how rhythmic networks are organized and operate [4]. An important principle to emerge has been the central role that the cellular properties of CPG neurons plays in rhythm generation (Box 1). Moreover, it is now apparent that the properties of CPG neurons are malleable and are strong influenced neuromodulators [4,6,42]. Many CPG networks are regulated by multiple neuromodulators that regulate neuronal excitability and synaptic transmission in a cell specific and state dependent manner. As such they have important roles in configuring the CPG output. Another key feature of these networks is the differential recruitment of CPG neurons during different motor behaviours. This is seen in the crustacean STG [43,44], in the leech locomotor network [45,46], and in the turtle spinal cord [47], where particular interneurons are active during both swimming and scratching while others are selectively recruited for each task. However, it is not clear whether the CPG is reconfigured in a task dependent manner or whether motor systems are comprised of multiple CPGs that use varying combinations of neurons from within the motor network for different behaviours.

Studies of simpler CPG networks have defined two general mechanisms for rhythm generation: pacemaker driven systems and mechanisms that rely on the emergent properties of the constituent neurons. The pyloric rhythm in the STG is largely driven by pacemaker neurons [48,49], and neurons with pacemaker properties have been identified in the pre-Botzinger complex that generates the inspiratory rhythm [50]. In other systems, oscillatory motor activity appears to be an emergent property that requires neuronal coupling between neurons [4,5]. Simple examples of the latter are the half-centre oscillator that drives swimming in *Clione* [3,51] and the beating of the heart in leech [42,46]. These oscillators depend on reciprocal inhibitory interactions between neurons, which are also thought to be important for the swimming CPG rhythm [5,7,

[52–53]. Nonetheless, the observation that rhythmic activity continues in the rodent cord when all fast inhibitory transmission is blocked [54], is but one indication of the rudimentary understanding we have of the cellular mechanisms that underlie locomotor rhythm generation in the vertebrate cord.

## Insights from the swimming CPG

The lamprey and amphibian spinal cord have provided key insights into the structure of the swimming CPG and the synaptic interactions that produce swimming movements [5–8]. Although the swimming movements of aquatic vertebrates differ markedly from the limb-dependent motor behaviours seen in terrestrial vertebrates, the overall organization and neuronal make-up of the locomotor system is remarkably conserved [5–8,13]. Because of this, the organization of the swimming motor system is likely to be a useful guide for elucidating the network structure of the locomotor CPG in terrestrial vertebrates that use their limbs to move. Studies in the lamprey have revealed four general classes of neurons as core elements of the swimming CPG [5]. These have functional and anatomical equivalents in the frog tadpole and in zebrafish, along with developmental, and presumably functional, homologues in birds and mammals (See Box 2 and Table 1).

## Locomotor circuits in terrestrial vertebrates

The transition from water to land based locomotion for vertebrate animals resulted in a marked change in the mode by which they move. Although sideways flexion of the torso provides an excellent mechanism for movement through water, this form of propulsion is largely ineffective on land or in air. Consequently, the majority of terrestrial vertebrates, aside from snakes, primarily use limbs for propulsion, with trunk movements often augmenting the gait by amplifying and prolonging movements. Weight bearing, postural changes and variations in limb placement also come into play with land based locomotion, and as a result the spinal circuitry required for limbed locomotion is more complex than that needed for swimming. One indication of this is the multiple spinal interneuron cell types that are known to contribute to the sensory reflex-dependent modulation of motor outputs in the cat [15–18]. This suggests that much of the neural diversity seen in the spinal cords of terrestrial vertebrates may result from the increased levels of sensory information that is used to inform and modulate the spinal motor machinery.

To date most of the studies examining the neural circuits involved in quadripedal locomotion the have been undertaken in cats and rodents [2–3,15–18,25,32–34]. Experiments in the cat have been key to understanding how spinal reflex pathways are organized and the role that sensory feedback plays in shaping movements. Several principles have emerged from analyses in the cat. First, sensory inputs are important for initiating and correcting the locomotor rhythm. Second, sensory inputs change the amplitude of the motor output and regulate phase changes during stepping and third, the transmission of reflexes and their actions vary with the step phase. Recordings from the cat spinal cord have also identified and characterized some of the interneuron cell types that are interposed in these spinal sensorimotor pathways [15–18]. More recent efforts to dissect the walking CPG have relied heavily on the isolated neonatal rat and mouse spinal cord preparation [13,32,36,39–40,58–60]. Each model system provides complementary information about the makeup and organization of the locomotor circuitry. Consequently, the value of merging these two rather disparate perspectives into a singular comprehensive view of the spinal locomotor circuitry cannot be underestimated. For example, the identification in mice of cell types previously characterized in the cat enables direct comparisons to be made between both systems. The genetic fate mapping of Renshaw cells and systems analysis of reciprocal inhibitory pathways in the mouse mark the beginning of such efforts [61–63].

## The mammalian locomotor CPG

The spinal CPG in walking mammals is a distributed network with centers at cervical and lumbar levels that control the forelimb and hindlimb, respectively [2,3]. The hindlimb CPG has been studied extensively, with early experiments in the cat showing that the lumbar and sacral spinal cord can elicit a normal pattern of walking activity when isolated from the rest of the CNS and from sensory inputs [19,21]. Smaller regions of the spinal cord can also generate coordinated motor activity. In the cat, the three segments from L6 to S1 retain the ability to generate a "normal" pattern of motor activity for ankle extensor and flexor muscles [21]. This and other studies indicate that CPG network for each limb comprises multiple interconnected modules that control the movement of each joint. Commissural and propriospinal connections secure cross coordination between both sides of the spinal cord, between the forelimbs and hindlimbs. CPG activity across multiple joints for each limb is also coupled.

The spatial arrangement of components of the hindlimb locomotor CPG network has been mapped in the rodent lumbar cord [64–66]. Evidence that the core CPG components are located in the ventral half of the spinal cord [66] are further supported by studies showing that activity-dependent markers are upregulated in lamina VII and lamina VIII during locomotor-related tasks [67–70]. Moreover, many ventrally-derived embryonic cell types with demonstrated roles in locomotion populate lamina VII and VIII in the adult cord [29,33,40,70], as do dI6 commissural neurons that arise in the dorsal half of the neural tube [71–72] (Figure 3).

## Molecular identification of locomotor interneurons

Our current ability to molecular identify and manipulate locomotor interneurons in mice grew out of efforts to elucidate the genetic program that controls neuronal patterning in the embryonic spinal cord. In addition to providing molecular signatures for different interneuron cell types, these studies laid the groundwork for characterizing and functionally analyzing spinal interneurons using molecular genetic approaches. Underpinning these genetic studies was the finding that neuronal identity in the spinal cord is primarily determined by the dual activities of two morphogen gradients that impart dorsoventral positional information to dividing neural progenitors in the ventricular zone [29,73]. Ventrally, the notochord and floor plate produce Sonic hedgehog [29–30], while dorsally, the epidermis overlying the neural tube and roof plate and secrete BMPs (Bone Morphogenetic Proteins) [73]. The opposing activities of Shh and the dorsally derived BMPs restricts the expression of "patterning" factors to spatially restricted subsets of ventricular zone progenitors (Figure 3), which in turn results the ventricular zone being subdivided into dorsoventral progenitor domains (eleven in mouse and chick) that generate different generic classes of embryonic neurons [31].

In the mouse dorsal alar plate, six progenitor domains generate early-born dI1-dI6 neurons, as well as two late-born classes of dorsal interneurons [31,71–72]. In the ventral half of the neural tube, five classes: "generic" motor neurons and four classes of putative core CPG interneurons, the so-called V0, V1, V2 and V3 neurons [29–31,40,74–81] are produced. Each of these spinal CPG interneuron classes exhibits a unique phenotypic signature. V0 neurons are commissural neurons that extend axons rostrally for 2–4 spinal cord segments in the embryonic cord [77–78]. In contrast, most V3 neurons are excitatory commissural neurons that extend a caudally-projecting primary axon [40] (M.G., unpublished observations). The V1 neurons, like their *Xenopus* and zebrafish homologues, are inhibitory interneurons [79–83] with axons that project ipsilaterally and rostrally [79,83]. The V2 neurons, which comprise a mixed population of glutamatergic V2a neurons and inhibitory V2b neurons [74–76], also project ipsilaterally, but preliminary studies suggests that they extend their axons caudally across multiple spinal cord segments (M.G, unpublished observations). In addition to the V interneuron classes, dorsally derived dI6 neurons are also likely to contribute to the spinal locomotor CPG [70–72].

Each neuron class appears to give rise to multiple neuronal cell types that share a number of common anatomical features. For example, newborn motorneurons can differentiate as either visceral or somatic motor neurons, with the latter acquiring distinct columnar and pool identities [29–30]. The V1 class is also diverse, being the source of two types of local circuit inhibitory neuron – Renshaw cells and Ia inhibitory interneurons 61¯62 – as well as one or more undefined inhibitory neuron subtypes. The V2, V3 and dI6 populations are also made up of multiple molecularly distinct cell types 40›[74] (T. Hendricks and M.G, unpublished observations), with each subtype likely to have specialized roles in locomotor control.

## Commissural interneurons: keeping both halves in step

Experiments in rodents and cats have demonstrated the importance of commissural connections for coordinated left-right limb movements [84–85]. Moreover, blocking fast inhibitory transmission results in the loss of left-right alternation and the production of a synchronous pattern of left-right motor activity in the isolated spinal cord [54]. Inhibitory commissural pathways therefore play critical roles in controlling left-right alternation during walking, much as they do in swimming [5–8,52–53,87–88]. V0 neurons are one of two classes of molecularly-defined inhibitory interneuron found in lamina VIII [70]. These commissural neurons are derived from progenitors that express the homeodomain transcription factor Dbx1, and Dbx1 is required for their development and commissural connectivity [78]. This observation was exploited to demonstrate that V0 neurons are necessary for proper coupling of the left and right hindlimb CPGs during “walking” [70], as isolated spinal cords from *Dbx1* $^{-/-}$ mice exhibited intermittent periods of synchronous “hopping-like” activity. Since periods of normal alternation still occur in the *Dbx1* mutant cord, the V0 neurons are not solely responsible for securing left-right alternation [70]. The dI6 neurons, which are inhibitory commissural neurons (T. Hendricks and M.G, unpublished), may also contribute to the crossed inhibitory pathways that secure left-right alternation.

Mice lacking the receptor tyrosine kinase EphA4, its ligand, ephrin B3 or the Nck adaptor protein also exhibit a loss of left-right alternation [89–91], with experiments in the isolated spinal cord pointing to an intrinsic spinal cord defect [90]. Because EphA4 is expressed in many spinal cord cell types, it has been difficult to define with any precision the changes in connectivity that underlie the hopping gait of these mice. Interestingly, many of EphA4$^{+}$ neurons in the cord are excitatory neurons that project ipsilaterally [92]. Eph–dependent signaling plays a prominent role in axon guidance, and it has been suggested that ipsilateral excitatory CPG neurons may either aberrantly cross the midline or make ectopic connections with commissural neurons in the *EphA4* mutant. Recently, it has been shown that V2a neurons are ipsilateral components of a commissural pathway that secures left-right alternation [39]. Since some V2a neurons express EphA4 [92], defects in V2a connectivity may contribute to the EphA4/ephrinb3/Nck phenotype.

Genetic approaches in zebrafish are now beginning to shed light on the commissural pathways that control swimming movements, which previously relied on pharmacological manipulations [52–53,88–89]. Mutations in the glycine receptor b2 gene (*glrb2*) that prevent glycine receptor clustering cause trunk muscles on both sides of the animal to contract synchronously 93. Likewise, fish lacking GlyT1 glycine transporter activity have enhanced glycinergic transmission and are unable to generate rhythmic swimming movements 94. Although these findings support a role for glycinergic commissural neurons in coupling the CPG networks in each half of the cord, both mutations affect inhibitory transmission non-specifically. To date it has not been possible to selectively inactivate inhibitory commissural neurons in zebrafish. Moreover, there are likely to be multiple glycinergic commissural neurons in swimming vertebrates: the commissural connections typically depicted in schematics of the swimming CPG (see ref. 5) probably derive from more than one cell type.

Excitatory neurons are also components of the locomotor commissural network, with the V3 neurons being the major class of excitatory commissural neurons in the mouse spinal cord [40]. V3 neurons play an important role in establishing a stable and balanced locomotor rhythm. Although largely dispensable for left-right alternation, they are important for the production of a symmetrical motor output from the spinal cord [40]. While homologues of V3 neurons in fish have not been identified, the VeMe and UCoD cells show marked similarities in their morphology and neurotransmitter phenotypes [96–97] (Figure 4). A class of excitatory commissural neurons in the zebrafish hindbrain, the spiral fibre neurons, can also regulate motor behaviors via descending reticulospinal pathways. The spiral fibre neurons form a circuit that controls fast turning movements and the escape reflex. In *space cadet* mutant fish, their axons no longer cross the ventral commissure to innervate Mauthner cells and inhibitory PHP interneurons that are presynaptic to Mauthner cells [95]. This leads to abnormal turning when the escape reflex is activated.

## Ipsilateral interneurons: the story so far

Three major populations and one minor population of genetically-defined interneurons in the embryonic and neonate mouse spinal cord are likely to be core constituents of the locomotor CPG. The V1 and V2b neurons generate inhibitory cell types, whereas the V2a and Hb9-expressing neurons are excitatory. The V1-derived neurons have been characterized in the most detail, with lineage tracing studies showing that Renshaw cells and Ia inhibitory interneurons are derived from this population [61–62]. However, these comprise less than 25% of the V1-derived cells, demonstrating that there are additional uncharacterized V1-derived cell types in the adult. Although molecular markers have been found that selectively mark the non-Renshaw cell, non-Ia inhibitory V1 neurons (M.G., unpublished observations), the neurons expressing these markers have not been correlated with any of the functional cell types previously identified in the cat, such as non-reciprocal Ia neurons or Ib inhibitory neurons.

The Gata2/3-expressing V2b interneurons are interspersed with V1 neurons in lamina VII [74–76,98]. These neurons differ from their V1 counterparts in that their primary axons project caudally (M.G., unpublished observations). Although the adult progeny of V2b neurons have not been identified, they may contribute to reciprocal inhibitory pathways, since disynaptic inhibition is still present in mice that lack V1 neurons [63]. The V2b cells are one likely source of these Ia inhibitory connections, thereby raising the intriguing possibility that certain physiologically-defined classes of spinal interneurons are more heterogeneous than previously thought and may be derived from more than one embryonic class of interneuron.

Less is known about the ipsilaterally-projecting excitatory interneurons in the rodent spinal cord, Of the three molecularly-defined classes that have been identified in ventral motor regions, only the Hb9 neurons have been characterized in detail [99–102]. $Hb9^{+}/VGlut2^{+}$ neurons are located medially in lamina VIII at lower thoracic-upper lumbar levels of the spinal cord. They are a small group of neurons, whose embryonic origin is not known, as most Hb9 cells in the spinal cord differentiate as motor neurons. Glutamatergic Hb9 neurons exhibit a number of cellular properties that suggest a role in rhythm generation, including rhythmic oscillations in membrane potential and pronounced post-inhibitory rebound when hyperpolarized [99]. Unfortunately, genetic tests to determine whether the Hb9 interneurons play a role in rhythm generation have been hampered by the expression of Hb9 in motor neurons and other spinal interneuron cell types.

A growing arsenal of genetic tools has facilitated a strong push to understand the contribution that these genetically-defined interneurons make to the locomotor CPG. Two ipsiltateral CPG interneuron populations have been analyzed so far. V2a neurons were selectively ablated in the mouse by selectively expressing the diptheria-toxin A subunit (DTA) in Chx10-expressing

neurons [39]. This resulted in a partial uncoupling of the left and right halves of the spinal cord. The resultant loss of left-right alternating activity indicates that the V2a neurons are ipsilateral components of a spinal commissural pathway that coordinates left-right hindlimb movements in mice. It is likely that the V2a neurons serve this function by providing excitatory drive to spinal commissural neurons, as V2 axon terminals contact a subset of V0 commissural neurons 39. Interestingly, the selective ablation of the V2a neurons also results in increased variability of the step cycle period and amplitude of the locomotor rhythm, suggesting these cells may also contribute to rhythm generation 39.

The function of the V1 neuron population in shaping motor outputs in the isolated spinal cord has been analyzed with three complementary genetic approaches [36]. The first used mice lacking the *Pax6* gene, since these animals exhibit developmental defects that lead to the selective loss of V1 neurons in the neonate spinal cord. V1 neurons were also ablated by crossing mice expressing Cre recombinase under control of the *engrailed 1* (*En1*) promoter with a mouse that conditionally expresses DTA, thereby driving the selective expression of DTA in V1 neurons. The third approach, also using the Cre-loxP system, involved conditionally expressing the receptor for allatostatin [103], an insect neuropeptide, in V1 neurons (Figure 5). Using the latter approach V1 neurons were acutely inactivated, thereby circumventing any compensatory changes that might occur during development. With all three approaches, the inactivation or deletion of the V1 neurons results in a marked prolongation of the step cycle [36]. Furthermore, preliminary results suggest that blocking neurotransmission in the V1 neurons following the expression of tetanus toxin (TeNT) also slows the motor rhythm (M.G, unpublished observations). These results suggest that these cells are critical for setting the speed of the locomotor rhythm. Indeed, adult *En1* mutant mice that have defects in V1 connectivity are unable to perform fast stepping movements [21]. The ability to acutely inactivate neurons is especially important when the loss of a gene or a genetically-defined cell type causes perinatal death or major developmental defects that preclude behavioral analyses, as is often the case for developmentally regulated genes. Such approaches will also facilitate functional analyses aimed at dissecting more complex motor behaviors in awake behaving animals [40,104].

It is still not clear how the V1 neurons regulate the speed of the locomotor step cycle. When cats are induced to walk fictively (without sensory feedback), Renshaw cells and Ia inhibitory interneurons show an overlap in activity with the motor neurons that they innervate active at the transition phase [105]. Although the inhibition provided by these cellls may contribute to burst termination during fictive walking, excitatory neurons that provide rhythmic drive from the CPG are likely to be the predominant target of V1 inputs. Intriguingly, the *Xenopus* homologues of the V1 neurons, aINs, are known to be differentially recruited during slow and fast swimming behaviors, being more active at faster swimming speeds than slower speeds [82]. At slow swimming speeds burst slow afterhyperpolarization mediated by $Ca^{2+}$ -activated $K^{+}$ currents may be the primary mechanism for burst termination [5]. At higher speeds, early phase inhibition to the CPG from aINs would likely facilitate burst termination, thereby increasing the frequency of swimming movements. However, the role that the aINs [82] and their zebrafish CiA counterparts [83] play in regulating the speed of swimming movements still needs to be tested.

## Evolutionary implications

The seemingly evolutionarily conserved role that the V1 neurons have in regulating the speed of the locomotor CPG rhythm indicates that certain neuronal modules may have been preserved between the swimming CPG and walking CPG. This reflects the close phylogenetic relationship between spinal neuron cell types in swimming vertebrates and their terrestrial counterparts, which is particularly apparent in the embryonic spinal cord [82–83,106–108] (Figure 4, Table 1). What remains to be determined is how the swimming CPG network has been

reconfigured to enable limb driven locomotion. The transition appears to have been gradual, with amphibians and reptiles exhibiting standing wave flexion movements of the axial body that are closely coupled to limb movements. It is still unclear whether the emergence of limb movements resulted from the reconfiguration of the swimming CPG at limb levels or the addition of a specialized module that controls limb flexor-extensor muscle groups. What is clear is that both forms of locomotion can be present in an animal at the same time, as in amphibia there is a gradual shift in the mode of locomotion during larval to adult metamorphosis [109].

The pectoral fins of teleosts possess primitive muscles groups that are homologous to dorsal (extensor) and ventral (flexor) limb muscle groups in walking vertebrates, and the progenitors for these muscles express the Lbx1 transcription factor [110], which is selectively expressed in the migrating appendicular muscle precursors of terrestrial vertebrates 111–112. These observations, together with studies in zebrafish showing "walking-like" movements of the pectoral fins during slow swimming 113, suggest that the neural substrates necessary for limbed locomotion are already present in bony fishes. Modeling studies are also supportive of this proposition [114]. For this reason, a detailed knowledge of the motor circuitry in swimming vertebrates should provide important insights into how the walking CPG in terrestrial vertebrates is organized.

## New tools, challenges and vistas

Until now, genetic analyses of the circuits in the spinal cord controlling locomotion have primarily utilized genetic and fluorescent tracers to label and trace particular neuronal cell types (Figure 5) [32--36,61–62,83,99–101,107]. In a few instances particular populations of neurons have been "silenced" either by expressing channels that attenuate neuronal activity or by blocking synaptic transmission [35,36,40] (Figure 5). These studies have revealed an underlying modular organization to the locomotor CPG, however, the relatively simple outputs measured in this way fail to reflect the richness and complexity of motor behaviors that are elicited during vertebrate locomotion. Nonetheless, an increasingly more sophisticated arsenal of molecular genetic tools that can be integrated with physiological and computational analyses of motor networks is coming on line. Among the tools are a variety of channels that can be used to either excite cells or to suppress neuronal activity and synaptic transmission [36,40,103–104,115–119]. Moreover, the spatial and temporal resolution of expression of these channels in vertebrate systems can be further refined with the use of intersectional approaches and tetracycline inducible promoters [37–38,120].

The developmental genes that have been analyzed to date are typically expressed in generic classes of neurons or in a spectrum of neuronal cell types. Future success will depend on identifying unique combinations of genes that mark specific cell types. This is already possible for Renshaw cells, since they are the only neurons in the cord that express both En1 and calbindin D28K [61]. The Hb9-positive neurons that express VGlut2[98–99] are also amenable to genetic manipulation. However, there is still a paucity of molecular markers that can be used to functionally subdivide these generic classes of spinal interneurons. Genetic screens to identify genes that are expressed in subsets of spinal interneurons are beginning to address this deficit [121]. The hope is that these efforts will identify new populations of locomotor interneurons and generate a coherent classification system for the many functional interneuron subtypes within the spinal cord.

New technologies are emerging for manipulating well-defined populations of neurons, and these should lead to a finer grained view of how the spinal locomotor system is organized and functions. Experimental approaches to assess the temporal activity of neurons within the locomotor CPG network during locomotion are also needed to complement ongoing genetic

and behavioral analyses. Technologies that allow the visualization of, and or recordings from, multiple neurons during particular motor behaviors are now possible [122–123], and are currently being used in the zebrafish embryo to study swimming behaviors [124–126]. With respect to the walking CPG, the neonate mouse spinal cord is perhaps ideal for opto-genetic analyses, due to its small size, partial transparency and the ability to genetically mark different neuronal populations using mouse molecular genetic techniques.

While the isolated spinal cord can provide important insights into the motor circuitry, such preparations do not allow one to assess the role that particular neurons play in postural control or complex motor behaviors. Many of the differentiated cell types that are present in the mature spinal cord that have specialized roles in sensorimotor reflexes cannot be assessed in the neonate spinal cord, as they are not fully differentiated and the sensory reflex pathways are still immature. Consequently, developing novel genetic approaches and behavioral tests that can be used for *in vivo* experimentation in adults will be necessary and can be guided by previous efforts in the cat. Other drawbacks with mice include difficulties in activating specific sensory reflexes and stimulating descending pathways, as well as recording from neurons in the intact cord. These issues are not insurmountable and efforts are underway to record from spinal interneurons in adult mice.

Another important issue is whether genetic markers represent a valid approach for identifying for different neuronal cell types in the cord. The use of genetics to manipulate subset of neurons in the cord is predicated on the hypothesis that the spinal motor circuitry is genetically "hardwired". Much of what we know about spinal cord development indicates that this is the case. However, the role that activity-dependent events play in shaping the locomotor network needs to be more closely examined. Spinal motor neurons are spontaneously active during embryonic development [127–129] with the networks generating this spontaneous activity maturing during embryonic development [128,129]. Embryonic neurons have low intracellular $Cl^-$ levels and consequently GABA and glycine transmission can be depolarizing or can facilitate excitation [130]. In midgestation rat embryos, depolarizing glycine and GABA synaptic currents drive synchronous patterns of motor activity [130]. This raises the possibility that inhibitory neurotransmitter pathways help configure the locomotor network during development. There is also evidence that altering neural activity can modulate neurotransmitter expression in neurons in the amphibian cord [131]. Such modulation appears to be part of a homeostatic mechanism that maintains network excitability in the spinal cord at appropriate levels during development [132], although its effect on behavioral outputs is not clear. Finally, activity-dependent changes in motor axon guidance have been reported in the mouse [133]. This, together with studies showing the topology of cutaneous sensory inputs to the dorsal spinal cord is refined by experience driven patterns of activity [134], points to activity playing a significant role in shaping the locomotor circuitry. Nonetheless, accurately assessing the contribution activity makes to circuit formation will require the use of genetic approaches to determine how these motor circuits are normally configured.

**Box 1. Properties of neurons that contribute to rhythm generation**

Neurons within a rhythmic circuit typically exhibit one or more of the following properties.

- Endogenous bursting: when isolated, some neurons fire in bursts spontaneously or in response to neuromodulators, thus functioning as pacemaker neurons [11,49].
- Postinhibitory rebound: when a neuron is hyperpolarized, the membrane potential reverses producing either a single action potential or a train of action potentials. Neurons that exhibit these properties in a network can contribute to rhythm generation [49].

- Spike frequency adaptation: these neurons will initially fire action potentials, but subsequently accommodate and cease firing
- Plateau potentials: these occur when transient excitatory inputs can lead to long lived depolarized states while transient inhibition hyperpolarizes the cell. Plateau potentials can contribute to bistability in neurons and oscillatory network activity. Interneurons and motor neurons in many motor systems exhibit plateau potentials [55–57].

The cellular, synaptic and modulatory mechanisms regulating CPG activity are discussed in two excellent reviews [45].

**Box 2. The four primary locomotor cell types found in the lamprey cord**

Four functional classes of neurons make up the swimming CPG in lamprey (see inset)

- Segmentally organized motor neurons that innervate each adjacent axial myotome.
- Glycinergic commissural interneurons (CINs) that project to the opposite side of the spinal cord. During swimming, inhibitory connections provide the mid-cycle inhibition that ensures that the axial muscles on each side of the body contract out of phase with those on the opposing side (excitatory commissural neurons of unknown function are also present in the lamprey).
- Ipsilaterally-projecting inhibitory L-interneurons (IINs) that provide inhibition to motor neurons and to glycinergic commissural neurons. Their exact role in swimming has not been defined.
- 4. Excitatory glutamatergic neurons (EINs) that project to all three other CPG neurons cell types. These cells, or a proportion of these cells, are rhythmically active and provide rhythmic drive to motor neurons and other CPG neurons during swimming. Excitatory commissural neurons are also present in the lamprey cord [86], however their function is not known.

**Summary**

The central pattern generator (CPG) networks that generate relatively simple motor outputs are ideal experimental models for circuit analysis

Locomotor CPGs in the ventral spinal cord function autonomously to generate repetitive patterns of oscillatory motor activity.

Recent progress has been made on identifying the neuronal components that make up the locomotor circuitry, with functional studies indicating the locomotor CPG has a modular structure.

The development and assembly of the locomotor CPG is regulated by a genetic program that operates in the embryonic spinal cord.

The merging of genetic analyses with systems approaches, coupled with new tools for imaging and regulating neuronal excitability provides the means for a comprehensive analysis of these circuits.

The emerging phylogenetic relationship between neurons in the vertebrate spinal cord is providing key insights into the structure and function of the spinal motor circuitry.

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## Biography

Martyn Goulding is a Professor at the Salk Institute for Biological Studies in La Jolla. After obtaining his Ph.D. from the University of Auckland, New Zealand, he moved to the Max Planck Institute for Biophysical Chemistry in Goettingen, Germany. There he worked in the laboratory of Peter Gruss studying the role of the Pax transcription factors in development. His current interests are focused on the development of sensorimotor circuits in the spinal cord and ascertaining how these circuits are organized and function at the cellular level. Web Link: http://www.salk.edu/faculty/goulding.html

## Glossary

| | |
|---|---|
| Alar plate | Dorsal region of the ventricular zone in the embryonic spinal cord |
| Central pattern generator | Network of neurons that autonomously generate rhythmic patterns of activity |
| Commissural neurons | Neurons whose axons cross from one side of the spinal cord to the other side |
| Fictive locomotion | Locomotion that is initiated in the absence of sensory feedback and descending control from the cortex |
| Lineage analysis | Techniques that allow progeny of a cell in the embryo to be traced |
| Proprioceptive Sense | Sense of the body: internal receptors (e.g. muscle spindles and Golgi tendon organs) provide information about the position of the limbs and muscle tension |
| Ventricular zone | Innermost layer of the embryonic spinal cord that contains dividing progenitor cells |

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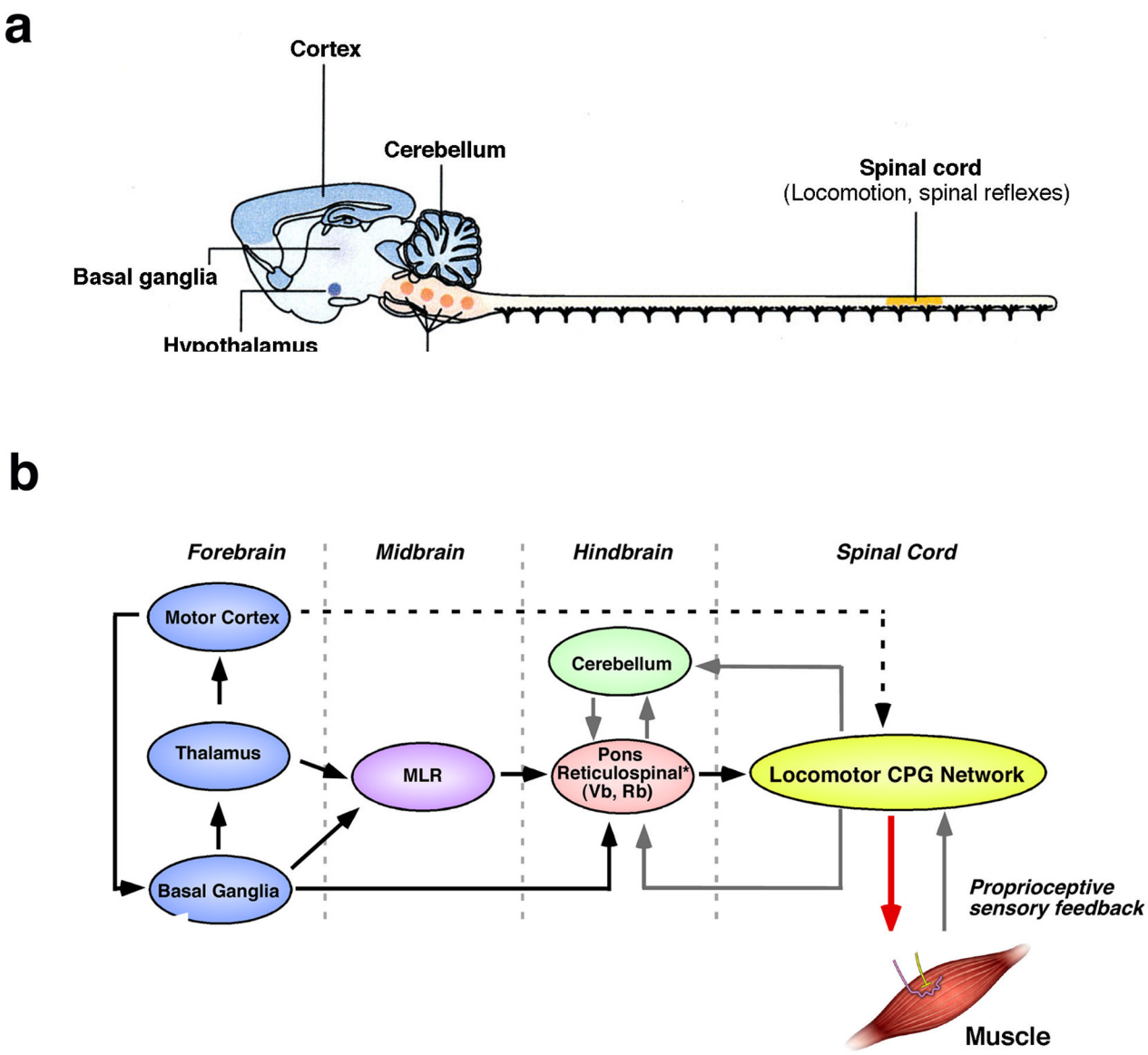


**Figure 1. Organization of the locomotor system in vertebrates**

**(a)** Schematic of the rodent central nervous system showing the neural structures that are part of in the motor pathways controlling simple behaviors such as mastication, respiration and locomotion. Adapted from Ref. [5]. **(b)** Motor pathways in aquatic and terrestrial vertebrates share a similar neuroanatomical structure. Local control of muscle movements is effected by pools of motor neurons in the spinal cord that are part of a dispersed locomotor CPG network. The motor commands are modulated by proprioceptive sensory feedback via sensory afferents. Descending reticulospinal, rubrospinal and vestibulospinal pathways control the locomotor network in the spinal cord, although the reticulospinal pathway is the primary pathway for intiating locomotion. The reticulospinal pathway can be activated by the mesencephalic locomotor region (MLR), which has inputs from the basal ganglia and thalamus. The cerebellum coordinates motor behaviors by mediating sensory and internal feedback and optimizing the motor pattern to the task at hand. It also coordinates spinal motor actions with the supraspinal motor pathways. Connections from the motor cortex refine and initiate motor

actions. The black lines indicate direct command pathways, the grey lines indicate feed-back pathways. VS, vestibulospinal; RbS, rubrospinal.

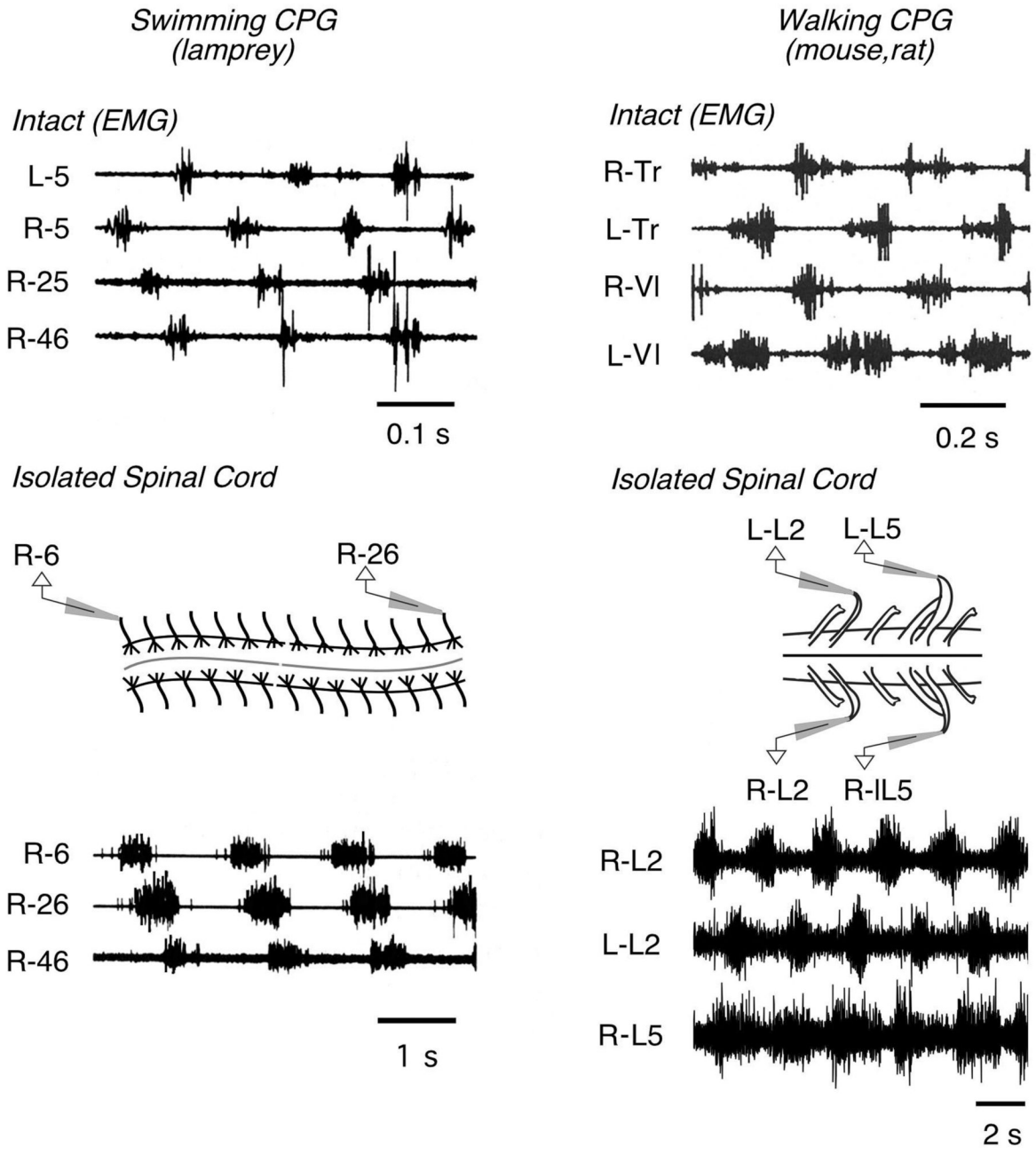


**Figure 2. Rhythmic motor patterns underlying vertebrate locomotion**

**(a)** Examples of spinal motor activity during swimming in the lamprey. (Top) Electromyograph (EMG) recordings of different myotomes at located at different axial levels. (Bottom) Ventral root recordings from the isolated spinal cord exhibit a slow pattern of rhythmic motor activity. N.B. The motor outputs of the intact animal and isolated spinal cord show the same patterns of motor coordination and segmental lag. **(b)** Walking motor behavior. (Top) EMG recordings showing muscle activity in the cat hindlimb. (Bottom) Isolates spinal cord preparation from P0 mouse. Electroneurogram (ENG) recordings from L2 and L5 ventral roots following the induction of walking by NMDA and serotonin (5-HT). The ENG traces give a measure of flexor-related (L2) and extensor-related (L5) motor activity. Adapted from Ref. [3].

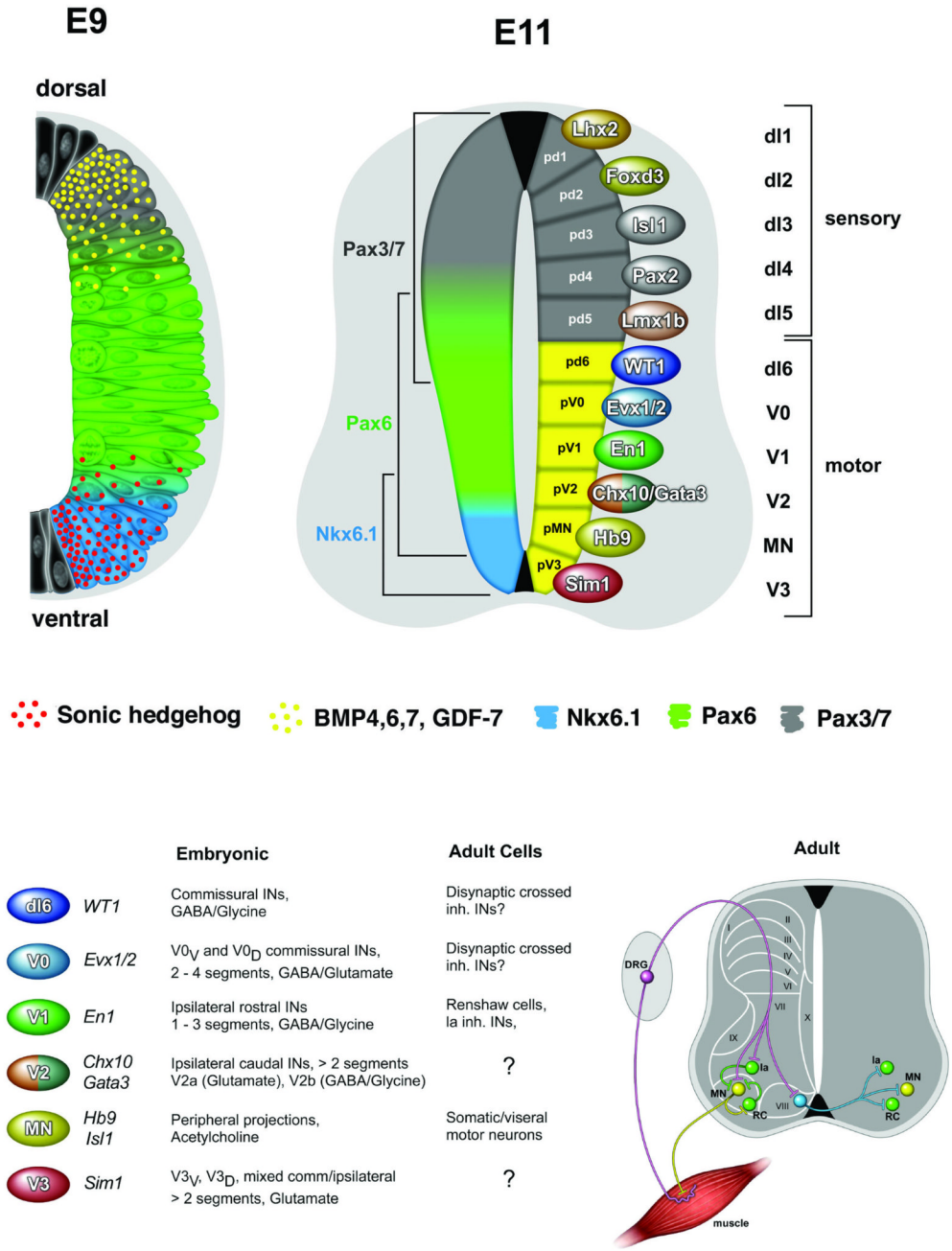


**Figure 3. Early development of the spinal cord**

**(a)** Schematic cross sections through the developing mouse spinal cord showing the patterning and specification of early spinal cord progenitors and their neuronal progeny. At E9, a gradient of Sonic hedgehog (red) ventrally and BMP/GDF7 (yellow) dorsally provide instructive positional signals to dividing progenitors in the ventricular zone. This leads to the restricted activation of patterning factors in discrete dorsventral domains, which are represented by Nkx6.1 (ventral), Pax6 (intermediate) and Pax3 and Pax7 (dorsal). At E11, eleven early classes of postmitotic neuron are present in the embryonic spinal cord. dI1-dI5 neurons that are derived from dorsal progenitors (grey) primarily contribute to sensory spinal pathways, while dI6, MN and V0-V3 neurons from ventral progenitors (yellow) are elements of the locomotor circuitry.

Some of the postmitotic transcription factors that mark each of the eleven early generic populations are indicated. **(b)** Six classes of embryonic neurons are proposed to give rise to the core elements of the spinal locomotor circuitry. The neurotransmitter phenotypes and axonal projections of these embryonic neurons are indicated along with some of the known adult cells types that are derived from each population. **(Right)** Schematic of the adult spinal cord showing the position and projections of somatic motor neurons (MN, yellow), V1-derived Renshaw cells (RC, green) and Ia inhibitory interneurons (Ia, green) and V0 commissural neurons (blue). The laminae of the spinal cord are indicated by Roman numerals.

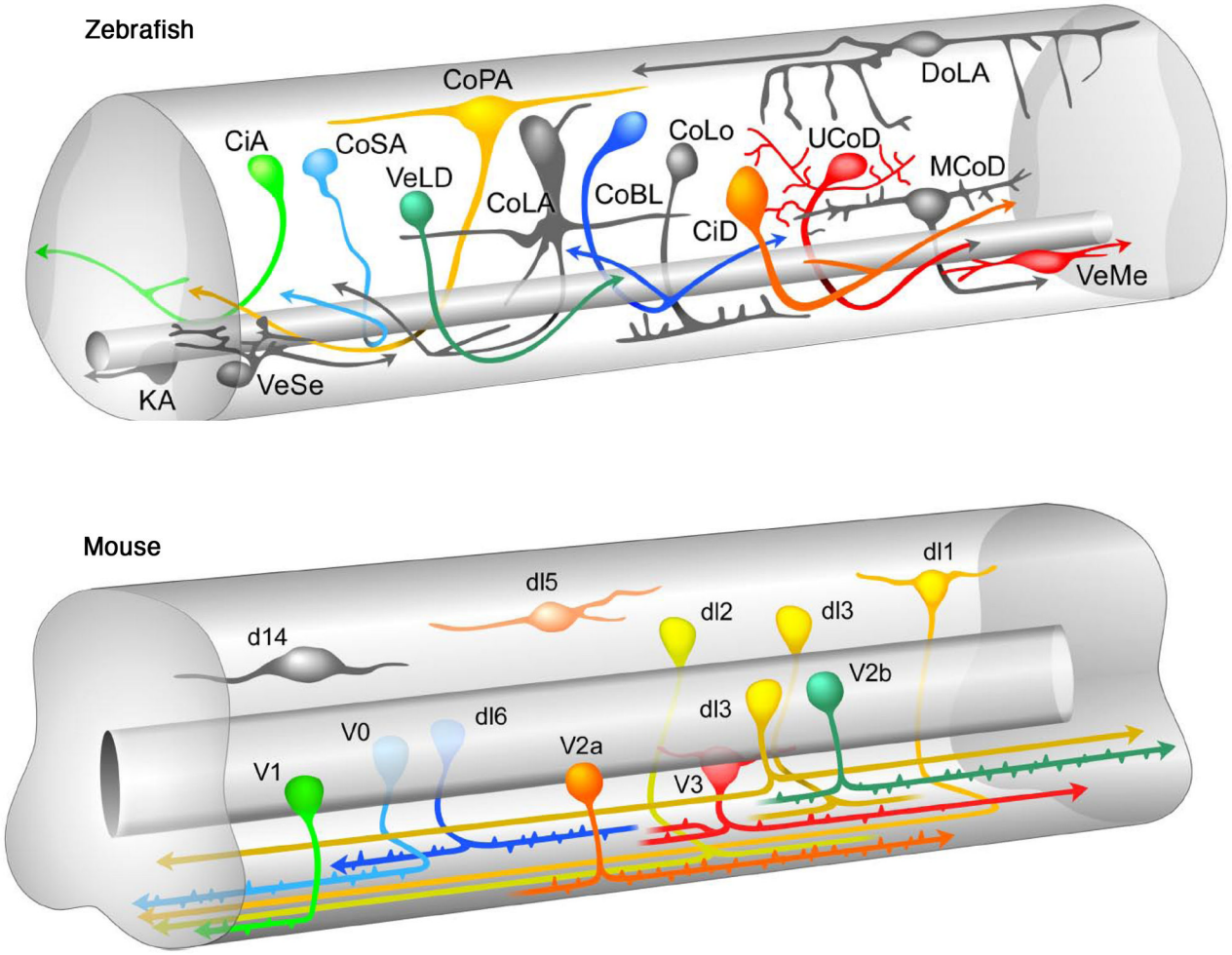


**Figure 4. Identified spinal interneurons in the embryonic mouse and zebrafish spinal cord**

Similar neuronal cell types are present in the embryonic spinal cords of aquatic and terrestrial vertebrates. The putative zebrafish homologues of V0, V1, V2 and V3 locomotor interneurons are indicated by the same colour. V0 and CoSA neurons (light blue), V1 and CiA neurons (light green), V2a and CiD neurons (orange), V2b and VeLD neurons (dark green), V3 and UCoD/VeMe neurons (red). Schematic of embryonic zebrafish spinal cord is courtesy of David McLean and Joe Fetcho (Cornell University).

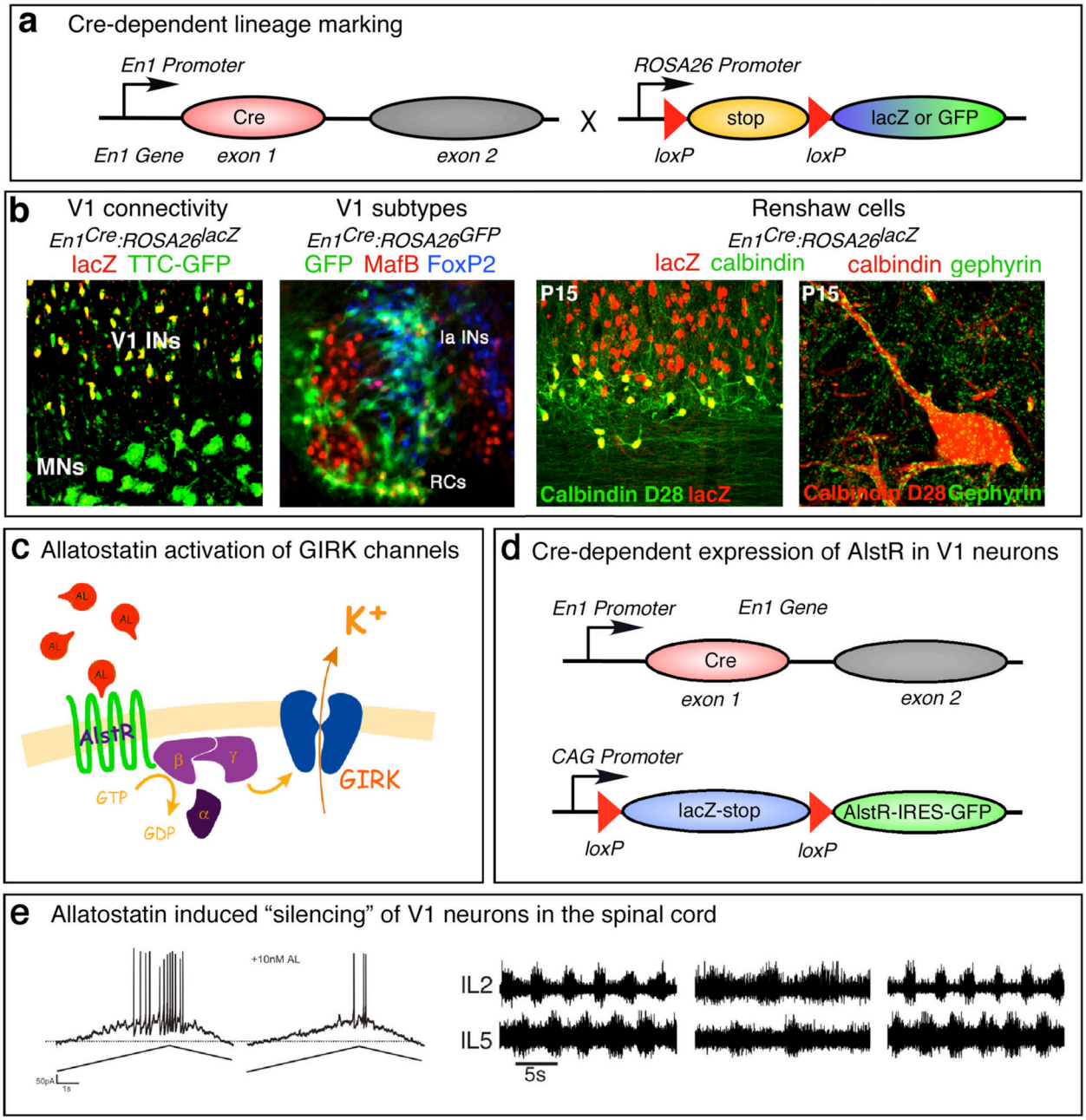


**Figure 5. Cre-dependent manipulation of V1 neurons in the mouse**

**(a)** Example of fate mapping and lineage tracing using the Cre-loxP system. Mice carrying an *En1*$^{Cre}$ allele, in which the first exon of the *En1* gene was replaced with sequences encoding Cre, were crossed with ROSA26 reporter mice that express either GFP or lacZ after removal of a loxP-flanked stop sequence. **(b)** Left panel: Intramuscular injections of the retrograde tracer TTC-EGFP in *En1*$^{Cre}$*:ROSA26*$^{lacZ}$ mice shows that V1 neurons are synaptically connected to motor neurons [61]. Middle left panel: V1 neuron subtypes differentially express MafB (Renshaw cells, red) and FoxP2 (Non-Ia/RC interneurons, blue). Right panels: Identification of Renshaw cells as V1-derived neurons. Renshaw cells are marked by the

expression of calbindin (left panel). Renshaw cells uniquely possess large gephyrin clusters on their soma and proximal dendrites (right panel).
**(c)** Schematic showing allatostatin-dependent activation of inwardly rectifying GIRK channels in mammalian neurons. **(d)** Cre-dependent expression of the allatostatin receptor in V1 neurons in mice. **(e)** Selective expression of AlstR-GFP in postmitotic V1 neurons at E11 results in a reduction in V1 neuron excitability (left) Allatostatin receptor-mediated silencing of V1 neurons in the locomoting spinal cord results in a slowing of the locomotor rhythm, which phenocopies the defect seen when V1 neurons are ablated (right; see Ref. [36] for details).

**Table 1**

**Putative phylogenetic relationship between identified neurons in the embryonic fish, amphibian and mammalian spinal cord**

aINs and CiA neurons appear to be equivalent to the inhibitory L- interneurons of the lamprey and V1 neurons in the mouse [21,61–62, 82–83]. In *Xenopus*, the dIN glutamatergic neurons appear to be the major source of ipsilateral excitatory input in the hindbrain and ventral spinal cord [7,135–136]. dIN neurons may be homologous to CiD neurons in zebrafish, that express Chx10 transcription factor [107]. CiD neurons, are rhythmically active during fictive swimming and provide the main source of on-cycle excitation to the swimming CPG [107,125–126]. Glycinergic inhibitory commissural interneurons play an essential role in generating these alternating outputs between each half of the spinal cord [5,52–54,87–88]. In *Xenopus*, the commissural interneurons (CINs) that mediate reciprocal inhibition have been characterized in some detail [87–88]. They typically fire in phase with ipsilateral motor neurons once per swimming cycle. There are multiple anatomically distinct populations of CINs, including CoPA, CoSA, CoLA, UCoD and CoBL cells in the zebrafish spinal cord [96–97,126]. Aside from their neurotransmitter phenotype, these cells are largely characterized molecularly and their functions in locomotion have not been described. CoBL and Evx2$^+$ MCoD neurons are both active during swimming [126]. CoBL cells are bifurcating glycinergic neurons. The excitatory commissural MCoD neurons are preferentially recruited during slow swimming movements. They appear to be necessary for slow swimming movements, but are dispensible for coordinating the left-right alternation of segmental motor neurons during fast swimming movements[126]. The inhibitory CINs, EINs and L-interneurons in the lamprey have not been molecularly characterized. The zebrafish homologues of V3 neurons, while not yet identified, may be VeMe and UCoD cells.

| Jawless Fish (Lamprey) | Bony Fish (Zebrafish) | Amphibian (Xenopus) | Mammal (Mouse) |
|---|---|---|---|
| Inhibitory commissural Interneurons (iCINs) | CoBLneurons<br>CoSA neurons | CINs | dI6 neurons (Lbx1+)<br>$V0_D$ neurons (Dbx1+, Evx1−) |
| Excitatory commissural Interneurons (eCINs) | MCoDneurons<br>UCoD neurons$^+$ | ?<br>? | $V0_V$ neurons (Evx1/2+)<br>$V3_v$ neurons (Sim1+) |
| Excitatory interneurons (EINs) | CiD neurons<br>VeMe neurons$^*$ | dINs | V2a neurons (Chx10+)<br>V3 neurons (Sim1+)<br>Hb9+ neurons |
| L-interneurons (ipsilateral inhibitory) | CiA neurons<br>VeLD neurons | aINs | V1 neurons (En1+)<br>V2a neurons (Gata3+) |

$^+$UCoD neurons are similar to commissural V3 INs (glutamatergic with descending axons).

$^*$VeMe cells also have a descending axons that may cross the ventral midline and are excitatory.