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Spinal Interneurons: Diversity and Connectivity in Motor Control

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Abstract

The spinal cord is home to the intrinsic networks for locomotion. An animal in which the spinal cord has been fully severed from the brain can still produce rhythmic, patterned locomotor movements as long as some excitatory drive is provided, such as physical, pharmacological, or electrical stimuli. Yet it remains a challenge to define the underlying circuitry that produces these movements because the spinal cord contains a wide variety of neuron classes whose patterns of interconnectivity are still poorly understood. Computational models of locomotion accordingly rely on untested assumptions about spinal neuron network element identity and connectivity. In this review, we consider the classes of spinal neurons, their interconnectivity, and the significance of their circuit connections along the long axis of the spinal cord. We suggest several lines of analysis to move toward a definitive understanding of the spinal network.

Contents

1. INTRODUCTION

Historically, the spinal cord has been divided into motor neurons, which project to muscles, sensory afferents of the dorsal root ganglia, and interneurons—that is, everything else. We emphasize that interneurons can be excitatory or inhibitory rather than purely inhibitory, as the term is applied in cortex. A long experimental record, primarily in cat and primate, of anatomical and physiological analyses in spinal interneurons has revealed tremendous diversity at every level. In the last 25 years, advances in mouse developmental genetics produced a new definition of diversity, with a dozen major classes of neurons identified by their transcription factor expression profiles. These genetic classes have proven useful in comparisons of neuron types across labs and across species, with the result that they are now the dominant means of identifying spinal neurons. However, in many cases it has been difficult to align functionally defined identities with genetically defined identities, leading to questions about what the best classification scheme really is. Because these interneurons are intermingled with each other, and many project long axons whose synaptic targets are not readily identified, there are significant technical challenges in defining the spinal circuit.

Here we first consider evidence for and against using genetics as the primary classification scheme for spinal interneurons, focusing on the motor circuits of the ventral spinal cord. We point to birthdate as a diversifying factor that may help explain the disparities between functionally and genetically identified spinal classes. Next, using the genetic framework to facilitate comparisons across species, we build a grid of connectivity among interneurons. This map demonstrates general concordance across species and highlights the need for further experiments to define interneuron connectivity. Finally, we consider an underexplored area: how interneuron identity and connectivity vary across the longitudinal axis of the spinal cord. Rostrocaudal coordination depends on longitudinal connections, yet our understanding of long-range circuitry is quite weak due to technical limitations. We discuss the importance of longitudinal connectivity for both axial- and limb-based locomotion.

2. SPINAL NEURON CLASSES: REALITY OR MIRAGE?

2.1. Classification Based on Morphology and In Vivo Recordings

The earliest successes of spinal neuron classification were interneurons that responded directly to stimulation of the dorsal or ventral roots, that is, the sensory afferents or motor neuron axons. For example, Renshaw cells were identified by their synaptic response to motor axon stimulation, indicating that they receive excitatory input from motor neuron collaterals. Renshaw cells inhibit homonymous motor neurons—that is, they inhibit the same motor pool that excites them (Alvarez & Fyffe 2007, Eccles et al. 1954, Moore et al. 2015). Conversely, the Ia inhibitory interneuron population was identified as responsive to dorsal root stimulation, specifically Ia afferents that report stretch; the Ia inhibitory interneurons inhibit the heteronymous motor pool—for example, if they are excited by flexor afferents, they inhibit extensor motor neurons (Eccles et al. 1956, Jankowska 1992). These two classes provided key early examples of feedback and reciprocal inhibition, motifs that would later be found throughout the nervous system.

Yet efforts at classification based on characteristics from in vivo recordings revealed serious limitations of the approach as well. The spinal cord mediates a wide variety of behaviors that, depending on the species, may include not just locomotion but scratching, reaching, jumping, flying, and postural adjustments. Berkowitz and colleagues (Berkowitz 2002, 2008; Berkowitz & Stein 1994) deployed the unique advantages of the in vivo turtle preparation to assay the activity of spinal interneurons during multiple distinct behaviors, including swimming and scratching. Notably, some spinal interneurons are recruited specifically during one type of movement or another, whereas the majority are multifunctional. In other words, physiological classification of interneurons during one behavior does not necessarily predict their activity—and thus their functional identity—in another (Berkowitz et al. 2010).

Another approach to classification is based on assessing the synaptic inputs to a neuron. However, spinal interneurons vary widely and unpredictably in the amount of synaptic input they receive from major descending pathways (Jankowska 2008). Attempts to cluster deep dorsal interneurons of the cat based on their sensory synaptic inputs also failed (Kohler et al. 2022). Even knowing the inputs onto a spinal neuron may not be definitive in predicting its recruitment. A synaptic connection assayed by stimulation under one set of conditions may perform very differently under another, such as an alteration in neuromodulatory tone (Jankowska 2001, Jha & Thirumalai 2020, Marder & Bucher 2001). In addition, two synaptic connections of similar amplitude may facilitate or depress during repeated stimuli, with different consequences for computation (Abbott & Regehr 2004). Anatomical approaches such as transsynaptic viral tracing provide a more holistic picture of connectivity but are also limited in interpretation because of the unknowns associated with synaptic transfer efficacy and neuronal death (Callaway & Luo 2015). Thus, synaptic input mapping has not provided a robust path for interneuron classification.

2.2. Classification Based on Genetic Identities

Developmental genetics ushered in a new era of neuronal classification in the spinal cord. Dorsoventral patterning conferred by morphogens creates discrete progenitor domains, identifiable by the expression of different transcription factors. In the current model, 11 progenitor domains give rise to the so-called cardinal classes (dorsal dI1–6, ventral V0–3, and motor neurons) making up all or nearly all of the spinal neuron network (Goulding 2009, Grillner & Jessell 2009). Because each of these cardinal classes expresses not only unique transcription factors but also a fairly coherent set of morphologies and transmitter identities (**Figure 1*a***), it seemed reasonable to think that the long struggle to classify spinal neurons was over.

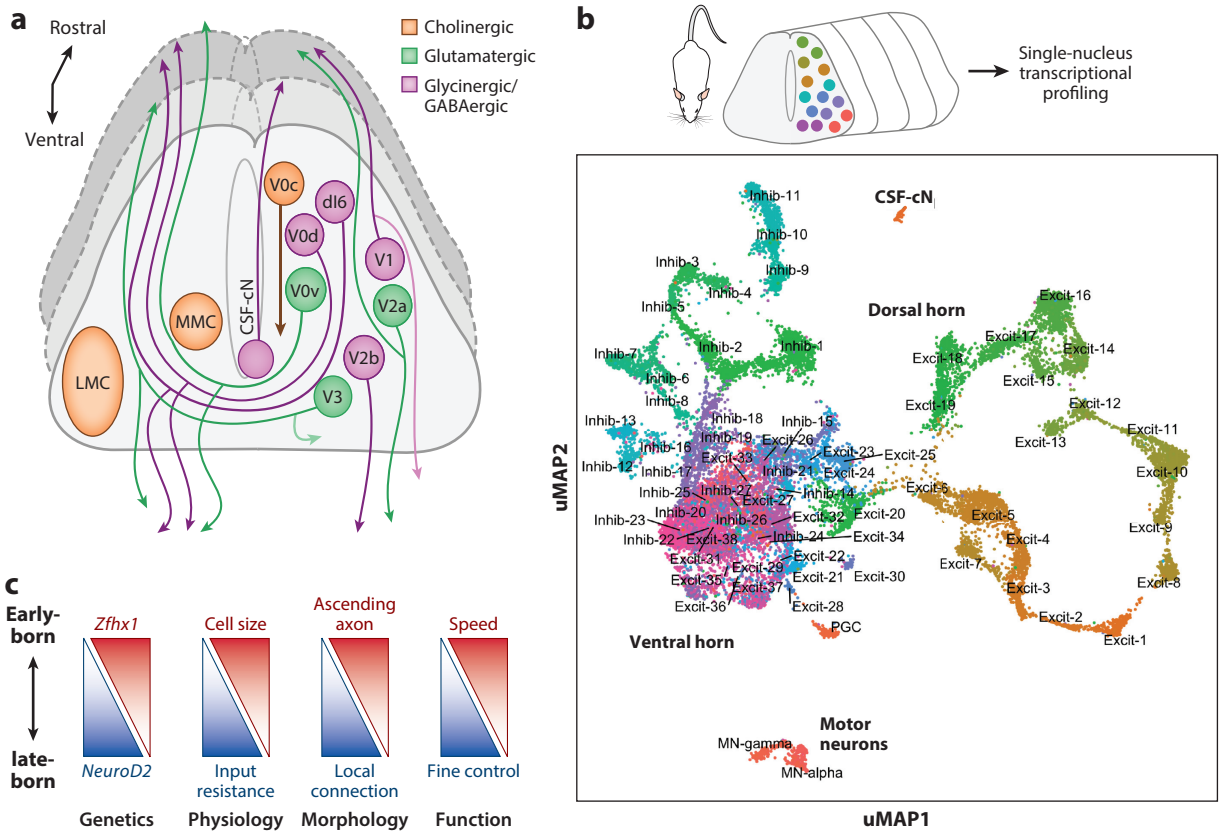

Figure 1

Diversity and overlap of ventral horn interneurons. (*a*) Schematic cross sections of the spinal cord show the cardinal classes of excitatory (cholinergic and glutamatergic) and inhibitory (glycinergic and GABAergic) interneurons and the primary motor nuclei. Soma positions in the ventral horn and axon lengths are not to scale. (*b*) Transcriptional profiling of spinal interneurons shows clear separation of dorsal nuclei but overlap of ventral horn neurons. (*Top*) Schematic of the experimental design. Mouse spinal cord tissue was used for single-cell RNA sequencing in a series of experiments that were merged to produce a harmonized atlas of neuron types. (*Bottom*) uMAP visualization plots of the 69 clusters from 19,353 neurons, color coded to depict location within the spinal cord. Dorsal horn neurons (*yellow* and *green*) form largely distinct clusters, whereas ventral horn neurons (*blue* and *purple*) are poorly separated. The exceptions are motor neurons and CSF-cNs, shown in red. Panel *b* adapted from Russ et al. (2021) (CC BY 4.0). (*c*) Schematic representation shows the correlation between birthdate and gene expression, physiological parameters, morphology, and function. Abbreviations: CSF-cN, cerebrospinal fluid–contacting neuron; LMC, lateral motor column; MMC, medial motor column; uMAP, uniform manifold approximation and projection.

And indeed, researchers have made significant strides using this classification scheme, demonstrating that genetic ablation of one class or another has distinct effects on limb movement, locomotor speed, or coordination (Butt & Kiehn 2003, Gosgnach et al. 2006, Lanuza et al. 2004, Talpalar et al. 2013, Zhang et al. 2008). Undoubtedly, developmental genetics now provides the dominant framework for research on spinal network function; these advances have been reviewed effectively elsewhere (Goulding 2009, Kiehn 2016, McLean & Dougherty 2015), and we do not cover them in depth here. However, in the last decade there have also been cracks in this framework. Some of the cracks have come from within. For example, while it was apparent early on that the V1 (*En1*$^{+}$) domain included the well-described Renshaw cells, they only represent approximately 5% of the V1 population (Alvarez et al. 2005). The slightly alarming implication was

that the V1 domain might contain 20 or more subclasses with completely distinct functions, and indeed, a later analysis subdividing V1 neurons based on soma location and transcription factor expression predicted approximately 50 subclasses (Bikoff et al. 2016). Similarly, the V2 domain is subdivided into excitatory (V2a, *Chx10/Vsx2*$^+$) and inhibitory (V2b, *Gata3*$^+$) classes, and targeted sequencing of the V2a population predicted 11 subclasses within it (Hayashi et al. 2018). Meanwhile, some genetic markers straddle these major classes (Delile et al. 2019, Dougherty et al. 2013), opening the possibility that entirely different classification schemes might be valuable. These results do not necessarily challenge the overall framework of developmental genetic markers for neuronal classification, but any initial optimism that spinal interneuron classes would be amenable to circuit-cracking rapidly breaks down in face of the prospect that there might be hundreds, rather than tens, of genetically defined neuron classes.

Still other limitations with the developmental genetics framework have become apparent when researchers try to map well-described functional neuron types onto their transcriptional identities. For example, the Ia inhibitory interneuron population, which supports flexor-extensor alternation, derives from two genetically distinct pools, the V1 and V2b classes (Alvarez et al. 2005, Zhang et al. 2014). Though V1-derived Ia inhibitory interneurons preferentially inhibit flexors and V2b-derived interneurons preferentially inhibit extensors, there is a great deal of overlap (Britz et al. 2015). Accordingly, one clear functional identity—the Ia inhibitory interneuron—maps onto two genetic identities. In concert with the demonstration that V1 neurons include many functional identities, genetic identity is insufficient to predict functional identity, and vice versa.

The era of massive single-cell RNA sequencing might have been expected to resolve these problems by allowing for unbiased classification of spinal neuron classes. However, to date, efforts at defining a spinal cord atlas based on sequencing have had mixed results. A notable study from the Levine lab (Russ et al. 2021) took on the challenge of integrating and comparing multiple spinal cord sequencing data sets (Baek et al. 2019, Häring et al. 2018, Hayashi et al. 2018, Rosenberg et al. 2018, Sathyamurthy et al. 2018, Zeisel et al. 2018) with the goal of identifying consistently separable classes. Some spinal interneuron classes, like the populations of the dorsal (sensory) horn, segregate cleanly in transcriptional data. Others are less distinctive, particularly the pools within the ventral (motor) horn (Russ et al. 2021) (**Figure 1*b***). By comparing sequencing results from embryonic and adult tissue, the authors further demonstrated that adult neurons with similar genetic profiles may arise from multiple progenitor domains. If so, then the genetics of the spinal cord may not prove as useful a guide as hoped in interpreting spinal networks and function.

By contrast, the value of genetic identification schemes has been much more apparent in simpler vertebrates such as zebrafish and, recently, tadpole. The zebrafish field transitioned rapidly from using morphology-based nomenclature to genetically based nomenclature, aided by the development of new tools for transgenesis. Neurons arising from each progenitor domain exhibit high homology to those in mouse, with key characteristics (transcription factor expression, axon trajectories, neurotransmitter expression) largely preserved. There appear to be fewer subclasses within each progenitor domain, simplifying analysis. Although the zebrafish spinal cord has not yet been sequenced in the same depth as that of mouse, various approaches have commonly revealed fewer than five subclasses per domain (Iglesias González et al. 2021, Satou et al. 2020). For example, the V2a population in zebrafish has been subdivided into just two subclasses, those with bifurcating or descending axons, each of which exhibits distinctive physiological characteristics in both larvae and adults (Menelaou & McLean 2019, Menelaou et al. 2014, Song et al. 2018).

These classes have aligned well with in vivo recordings from targeted neuron types during fictive (paralyzed) behaviors. While the repertoire of behaviors in aquatic model systems is narrower than in limbed animals, these animals nonetheless perform several distinct forms of locomotion, including swim, struggle (backward swim), and escape (rapid, large-amplitude bends). As in turtle,

individual neurons can participate in just one or many of these behaviors. Combining reconstructions with genetic identification of glycinergic neurons revealed that subtypes of morphologically distinct inhibitory interneurons are associated with different sets of behaviors: Some are specific for escape, some for struggle, and some for multiple behaviors (Liao & Fetcho 2008). Later work successfully mapped most of these morphological identities onto genetic classes: For example, the inhibitory CoLo neurons, exclusively active during escape, are a *Dmrt3a*$^+$ subclass of the dI6 domain (Kishore et al. 2020; Satou et al. 2009, 2020).

2.3. Neuronal Birthdate as Diversifying Factor

Though there appear to be fewer discrete spinal neuron subclasses in zebrafish, a large body of work has identified a key diversifying factor within each class: neuronal birthdate. Motor neurons are born in a stereotyped sequence, with primary motor neurons that innervate fast muscle being born first, and secondary motor neurons innervating slow muscle being born later. This pattern of early-born neurons for fast motor control and later-born neurons for slow motor control appears to extend to V1, V2a, and V0d neurons (Kimura & Higashijima 2019, McLean & Fetcho 2009, Picton et al. 2022, Satou et al. 2020) as well as to descending circuitry (Liu et al. 2022, Pujala & Koyama 2019). Intrinsic physiology and morphology of motor and premotor populations exhibit birthdate-related gradients as well: Early-born V2a neurons typically have longer axons and lower input resistance than do late-born neurons (McLean & Fetcho 2009, Menelaou et al. 2014). Similarly, large, low-input resistance motor neurons mediate the fastest muscle contractions (Heckman & Enoka 2012).

This work sets up a striking hypothesis: that the difficulty in parsing mouse spinal neurons into genetically defined subclasses may arise from the fact that protein expression aligns with birthdate and other associated properties (morphology, intrinsic physiology, axon length, rostrocaudal position) as much as with progenitor domain identity (**Figure 1*c***). In other words, the smear of genetics could reflect a continuum of birthdate-associated properties within each domain, with the result that, for example, a late-born V1 neuron may have as much in common with a late-born V2b neuron as with an early-born V1 neuron.

Recent work in mouse has built upon these extensive results in zebrafish to identify birthdate-associated differences in both morphology and gene expression patterns. Key work from the Zhang lab (Blacklaws et al. 2015; Borowska et al. 2013, 2015; Deska-Gauthier et al. 2020) showed that mouse V3 neurons are born sequentially over embryonic days 9–13 and that this birth order correlates with their diversification. Early-born V3 neurons migrate dorsally and laterally and exhibit characteristics common to early-born zebrafish spinal cord neurons: lower input resistance and larger cell bodies, higher spike frequency adaptation, and longer ascending axonal projections. In contrast, late-born V3 neurons remain medially near the progenitor zone and have opposite physiological properties. Recently, transcription factors aligning with early- and late-born populations have been used to segregate early- and late-born spinal neurons across progenitor domains (Delile et al. 2019), and indeed the early-born populations are the source of most long ascending axonal projections (Osseward et al. 2021), just as in zebrafish V0 and V2a neurons (Menelaou et al. 2014, Satou et al. 2012). Overall, these results support the idea that the developmental sequence produces a smear or continuum of properties overlaid onto the underlying structure of progenitor domains (**Figure 1*c***).

As a consequence, researchers looking for strong genetic signatures of every small subpopulation may be stymied, but the implications for sequential, speed-related circuit development are very exciting. The next vital question is whether the tight relationship between birthdate and movement speed identified in fish will carry over to mammalian/tetrapodal circuitry as well. There

are indications in mouse ex vivo calcium imaging that the population of neurons recruited during faster locomotion shifts more laterally compared to those recruited in slower locomotion (Rancic et al. 2020), consistent with the idea that early-born, more laterally positioned neurons mediate faster movements. Similarly, ascending V3 neurons are preferentially recruited by fast locomotion (Zhang et al. 2022). As researchers become able to carry out more multielectrode recordings in vivo, there will be improved opportunities to evaluate the idea of speed-dependent maps in tetrapods. Developing approaches to identify genetic neuron class during in vivo recordings in other animals besides zebrafish will aid in addressing this question.

Thus, while most spinal cord researchers would support the value of classifications based on progenitor domain and molecular markers, identification of spinal neurons will clearly be a prolonged struggle. We suggest that the spinal cord field could benefit from a collective effort to create a common reference frame, much as the cortical interneuron field did (Ascoli & Group 2008). In light of the fact that the role of developmental sequence in interneuron diversification was first identified in zebrafish, we also suggest that this collective classification effort should include perspectives from researchers working on a variety of organisms. In the remainder of this review, using the genetic framework to facilitate comparisons across species, we consider what is known about connectivity among spinal interneurons and their organization within the cord.

3. INTERCONNECTIVITY IN THE VENTRAL SPINAL CORD

In part due to the many challenges in interneuron identification, there is a striking lack of knowledge about connectivity among those interneurons. To date, much work has focused on connectivity onto motor neurons. Yet computational models that can recapitulate behavioral output rely on synaptic connectivity among interneurons. This is true in models that include nominal categories of interneurons (Grillner et al. 2007), models that include identified categories of interneurons (Danner et al. 2017, Roussel et al. 2021), and models that dispense with directed structure altogether (Lindén et al. 2022). Despite the clear functional significance, there are very few methodical analyses of connectivity among interneuron classes (Haque & Gosgnach 2019). An integrated map of interneuron connectivity will not only reveal areas that need investigation but also steer new avenues of inquiry into assembly of spinal networks, interaction of different functional modules, and comparisons across species. Here we tabulate and consider the relationships among ventral horn interneurons as far as they have been demonstrated through physiological, anatomical, or viral tracing approaches.

3.1. Tabular Depiction of Connectivity

To visualize circuitry across ventral spinal interneuron populations, we developed a grid of known synaptic connectivity (**Figure 2**). The table encompasses data from genetically tractable vertebrates that rely primarily on axial-based locomotion (zebrafish, tadpole) or limb-based locomotion (mouse), although we note that data from mouse may well include axial circuitry, and one zebrafish citation comes from the fin circuit (Uemura et al. 2020). We have not included the V2c/V2s (Gerber et al. 2019, Panayi et al. 2010) population, nor have we attempted to separate out subclasses within each of the major classes, because of the limited connectivity data available. Cerebrospinal fluid–contacting neurons (CSF-cNs) are included due to their ventral origin, although they are thought to serve a largely sensory function and therefore likely receive little synaptic input from intraspinal sources. Some of the connectivity depicted here obscures diversity within subclasses. For example, within the dI6 class, the *WT1*$^{+}$ subclass does not form synaptic connections

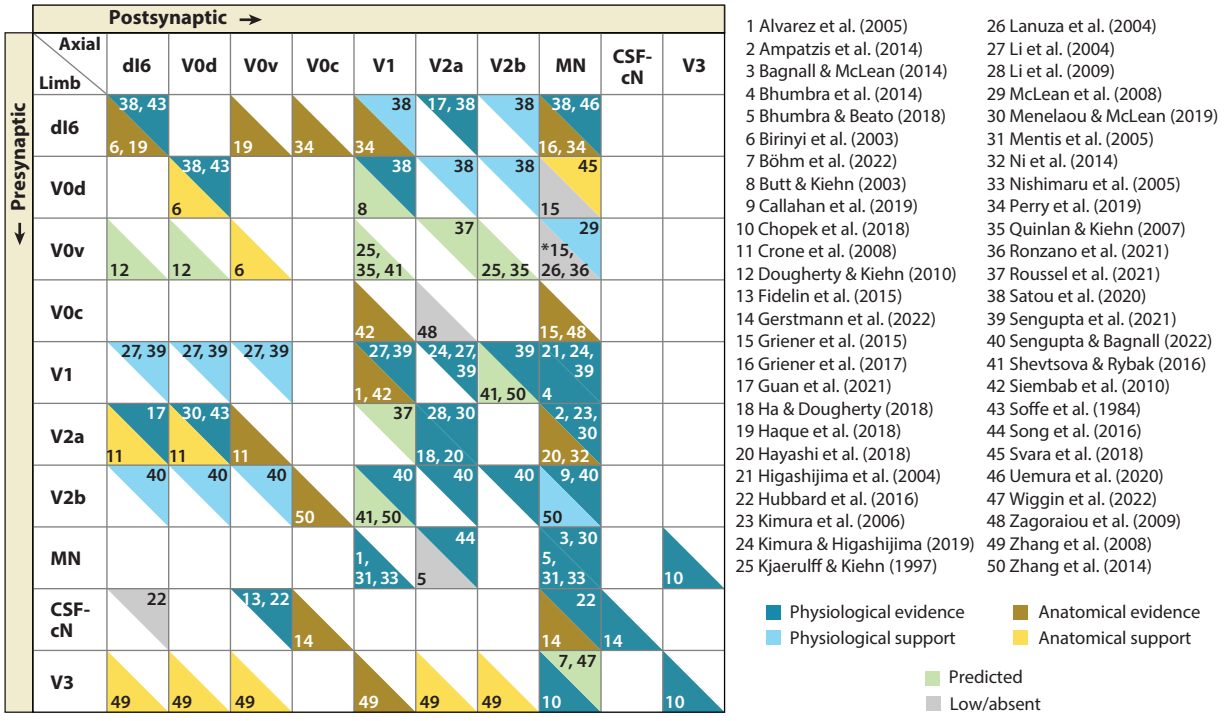

Figure 2

Survey of monosynaptic interconnectivity among spinal neurons of the ventral horn. Each square depicts connectivity between a given presynaptic and postsynaptic population. The upper right triangle in each square indicates data from animals that rely on axial locomotion (zebrafish, tadpole), while the lower left triangle indicates data from animals with limb-based locomotion (mouse). "Evidence" denotes connectivity between neurons with firm genetic identification, whereas "support" indicates some uncertainty regarding the specific identities of one of the populations, such as commissural inhibition from either dI6 or V0d. Numbers indicate the appropriate reference(s). Where there is both physiological and anatomical evidence for a connection, we have given preference to physiological data. An asterisk indicates conflicting evidence. "Predicted" denotes connections inferred from computational modeling or indirect experimental evidence. Abbreviations: CSF-cN, cerebrospinal fluid–contacting neuron; MN, motor neuron.

with motor neurons but instead with V0v and dI6/*Dmrt3*$^{+}$ neurons (Haque et al. 2018, Perry et al. 2019). Additionally, this grid is unable to represent what is known about longitudinal connectivity. For example, CSF-cNs inhibit other CSF-cNs located rostrally but not caudally (Gerstmann et al. 2022). We discuss rostrocaudal connectivity further in Section 4.

Several observations are apparent. First, there is widespread connectivity between interneuron populations. The field's focus on connectivity to motor neurons has paid off, as the column labeled "MN" in **Figure 2** is well populated with information, but the rest of the grid begs for more exploration.

Second, in most cases where there is evidence from both zebrafish and mouse, connectivity is similar. Owing to the respective technical advantages of each preparation, the best evidence for connectivity commonly comes from physiology in zebrafish and anatomy in mouse. Nonetheless, concordance is fairly high, especially in the well-explored territory of V2a and motor neurons circuitry. In fact, some connections predicted in limb circuits have been best supported by research in zebrafish. Finally, commissural connectivity that could underlie behavioral transitions, such as speed-dependent changes in gait, is only beginning to be quantified. These observations are discussed below.

3.2. Broad Connectivity Among Interneurons

Across organisms, connectivity among genetically defined classes appears widespread (**Figure 2**). Only in rare cases do we find evidence for the absence of a particular class of connections (see the gray shading in **Figure 2**). This observation could indicate that most interneuron classes are connected with each other, derive from underreporting of negative results, and/or reflect understandable hesitation to claim definitively that a connection does not exist. While it is difficult to ascertain the absence of connectivity, the fact that so many connections are already supported by literature indicates a complex set of circuit interactions.

Determining whether connectivity is truly broad, or in fact more limited, will have implications for our understanding of the spinal circuit. Several models of spinal networks rely on segmental oscillators and connectivity that couples or decouples these oscillators (Danner et al. 2017). However, the presence of segmental oscillators remains under debate (Hagglund et al. 2013, Wiggin et al. 2012). Alternatively, models invoking widespread inhibitory and excitatory connectivity recapitulate basic properties of locomotion (Dale 2003). Broad, balanced excitatory and inhibitory connectivity among interneurons is key to dynamical systems models of rhythmic oscillations (Lindén et al. 2022). These models of connectivity may not be mutually exclusive and could work in conjunction to support the wide repertoire of movements that animals exhibit. One possibility is that although the cardinal interneuron classes are broadly connected, as depicted in **Figure 2**, their subclasses have more restricted connectivity (Chopek et al. 2018, Menelaou & McLean 2019, Perry et al. 2019). Broad connectivity might be a hallmark of networks for fast movements, whereas limited connectivity might be the rule in networks for slower, precision movements (see Section 3.4). Analyses distinguishing connectivity in the context of speed will be vital in constraining computational models of the spinal circuit.

3.3. Conservation of Circuit Motifs

Perhaps the best-studied elements of the spinal network, V2a and motor neurons, show fairly strong homologies of connectivity across species. V2a neurons are the dominant source of ipsilateral excitation to motor neurons (Cangiano 2005, Grillner 1985, Ljunggren et al. 2014, Rancic & Gosgnach 2021). V2a neurons abundantly contact motor neurons as well as other V2a neurons, forming strong feedforward excitatory networks in both mouse and fish (Ampatzis et al. 2014, Ha & Dougherty 2018, Hayashi et al. 2018, Menelaou & McLean 2019, Menelaou et al. 2014, Ni et al. 2014). Data from both species show that V2a neurons excite commissural neurons (Crone et al. 2008, Guan et al. 2021, Menelaou & McLean 2019). Similarly, motor neurons are synaptic targets of almost all ventral horn populations in both mouse and fish.

One exception to these observed homologies is feedback from motor neurons to V2a neurons, which occurs via retrograde electrical synaptic connections in fish (Song et al. 2016) but is absent in mouse (Bhumbra & Beato 2018). The significance of this feedback is not yet clear. In mammals, it may have been replaced by motor neuron collateral excitation onto spinocerebellar neurons of as-yet-unknown genetic identity (Chalif et al. 2022) or other collateral targets. Some crucial elements of mammalian circuits, such as motor neuron feedback to V1 neurons, have not yet been demonstrated in zebrafish.

Interestingly, a motif of reciprocal inhibition appears to be similar across species. Flexor-extensor alternation is implemented by reciprocal inhibition of antagonistic motor pools (Eccles et al. 1956). Mutual inhibition between Ia inhibitory interneurons (Baldissera et al. 1987, Jankowska 1992, Pratt & Jordan 1987, Wilson et al. 2010) is predicted to be critical for enforcing alternation (McCrea & Rybak 2008) (see the sidebar titled Coincident Inhibition in Spinal Motor Circuits). Yet reciprocal inhibition between V1 and V2b neurons, which are identified as Ia

COINCIDENT INHIBITION IN SPINAL MOTOR CIRCUITS

Coincident inhibition in the cerebral cortex regulates network parameters like synaptic gain, dynamic range, and spike synchrony, all of which are crucial for computation (Isaacson & Scanziani 2011). Though the spinal cord field has traditionally focused on inhibition arriving out of phase with excitation as a means of enforcing alternation, motor neurons also receive inhibition coincident with excitation (Berg et al. 2007, Kishore et al. 2014). The likely sources of this coincident inhibition are V1 and V2b neurons, which are inhibitory and project ipsilaterally. V1 neurons fire action potentials at the falling phase of local motor neuron activity, consistent with their function of motor burst termination (Kimura & Higashijima 2019). In contrast, V2b neurons are active during the rising phase of excitation, leading motor neuron activity (Sengupta & Bagnall 2022), and may be poised to modulate motor neuron gain. Whether these or other classes of neurons may provide temporally distinct inhibitory influence on the rising and falling phases of motor neuron activity in tetrapods remains to be seen.

inhibitory sources (Alvarez et al. 2005, Zhang et al. 2014), has not yet been demonstrated in mouse. In zebrafish, our lab has recently shown that V1 neurons inhibit rostrally located V2b neurons, while V2b neurons inhibit caudally located V1 neurons (Sengupta & Bagnall 2022, Sengupta et al. 2021), just as might be expected in flexor-extensor circuits. Therefore, this classic motif of reciprocal inhibition in limb control may in fact derive from ancestral circuits for axial control.

An exciting research direction that may help bridge axial and limb circuits is analysis of the spinal architecture governing fins. The gene regulatory networks defining motor neurons that innervate fin and limb muscles are genetically conserved (Jung et al. 2018), suggesting that limbed locomotion evolved from undulatory swimming in aquatic vertebrates. Recent work in zebrafish has identified a population of dI6 neurons specifically targeted to fin abductors (Uemura et al. 2020), the homologs of limb extensors. These and similar types of analyses may help to determine which elements of appendage control are shared across species.

3.4. Interneuron Connectivity Underlying Changes in Speed

Subsets of spinal cord interneurons are differentially recruited during different speeds of locomotion, different gaits, and distinct movements such as turns. These modulations of motor output are likely to be triggered by changes in descending inputs (Cregg et al. 2020, Usseglio et al. 2020) but implemented by shifts in spinal interneuron activity. One mechanism for these shifts is that different sets of interneurons exhibit varying degrees of excitability and hence are recruited differentially with increasing excitatory drive from descending pathways (Shevtsova et al. 2015). This mechanism has been experimentally supported in adult zebrafish, where connectivity among interneurons and motor neurons is organized by speed, aligning passive and active properties (Ampatzis et al. 2014). Even within fast populations, V2a neurons show subtype-specific connectivity (Menelaou & McLean 2019, Song et al. 2018). Speed-related recruitment of V2a neurons is found in mice (Dougherty & Kiehn 2010; Zhong et al. 2010, 2011), but associated patterns of downstream connectivity are not yet known. Fast motor neurons themselves are preferentially interconnected (Bhumbra & Beato 2018), perhaps a mechanism for improving the synchrony in faster muscle contractions.

Interestingly, switches to faster locomotor speeds also appear to rely on selective inhibition of slow-speed interneurons. For example, in zebrafish, V0v neurons and slow V2a and motor neurons are selectively inhibited at fast speeds of locomotion (Kimura & Higashijima 2019,

McLean et al. 2008). V1 neurons recruited at fast speeds supply this selective inhibition to slow V2a and motor neurons (Kimura & Higashijima 2019). Whether a similar V1-mediated shift occurs in limb circuits remains unknown.

In many tetrapods, a change in speed is accompanied by a gait shift from left-right alternation to synchrony. Commissural excitatory (V0v, V3) and inhibitory (dI6, V0d) neurons are vital to left-right coordination (Buchanan 2001, Crone et al. 2008, Dale & Roberts 1985, Grillner 2003, Lanuza et al. 2004, Zhang et al. 2008). The switch to synchronous gait appears to be mediated by different populations of commissural neurons (Bellardita & Kiehn 2015, Talpalar et al. 2013). The selective inhibition hypothesis predicts suppression of left-right alternation (Dougherty & Kiehn 2010) at fast speeds of locomotion as a mechanism. This idea receives some support from the observation that mutations in *Dmrt3* (a dI6 subpopulation) can produce faster gaits that retain left-right alternation (Andersson et al. 2012).

However, the intermediary neurons that facilitate cross talk and selective inhibition between different gait modules remain unknown. Modeling studies predict that these would be ipsilaterally projecting inhibitory populations, specifically V1 neurons (Shevtsova & Rybak 2016), based on the observation that V2b-deficient hemicords have impaired flexor-extensor alternation (Zhang et al. 2014). However, to date, only *Dmrt3*$^{+}$ dI6 neurons have been shown to contact the Renshaw cell subpopulation of V1 neurons (Perry et al. 2019). Despite ample evidence supporting polysynaptic input from V0 neurons to motor neurons (Butt & Kiehn 2003, Griener et al. 2015, Kjaerulff & Kiehn 1997, Quinlan & Kiehn 2007), evidence regarding the specific intermediary targets of the V0d and V0v populations is quite weak. Even V0v connectivity onto motor neurons is disputed, with references supporting (Lanuza et al. 2004), disputing (Griener et al. 2017), or possibly supporting (Ronzano et al. 2021) this connection in mouse, and there is modest physiological support from a presumed V0v class, the MCoDs (multipolar commissural descending neurons), in zebrafish (McLean et al. 2008). Our understanding of commissural connectivity lags behind that of ipsilateral connectivity due to technical challenges, including the loss of many commissural pathways in slice preparation and the fact that each of these classes can only be identified through intersectional genetics. Defining commissural connectivity, especially in circuits for fast and slow movements, will be vital to understanding spinal network function.

4. LONGITUDINAL ORGANIZATION OF SPINAL CIRCUITS

Coordination in the longitudinal or rostrocaudal axis is essential for locomotion (Bonnot et al. 2002, Chevallier et al. 2008, Kozlov et al. 2009, Tunstall & Roberts 1994, Wolf et al. 2009). Studies from different species have shown that rhythmogenesis in the spinal cord is distributed (Deliagina et al. 1983, Grillner 1985, Hagglund et al. 2013) but also distinct in rostral versus caudal parts (Cazalets et al. 1995, Kjaerulff & Kiehn 1996, Wiggin et al. 2012). Two factors are thought to support smooth propagation of activity from head to tail: a rostrocaudal gradient in excitatory drive (Kozlov et al. 2009, Tunstall & Roberts 1994) and localized inhibition (Perrins & Soffe 1996, Sengupta et al. 2021). Here we review the evidence that both the composition of spinal interneurons and their circuitry vary from rostral to caudal levels, along with implications for locomotor function.

4.1. Genetic and Cellular Variation in the Longitudinal Axis

The spinal cord is patterned by *Hox* gene expression along the rostrocaudal axis, with distinct sets of *Hox* paralogs expressed at cervical, thoracic, lumbar, and sacral levels. *Hox* genes appear to dictate not just rostrocaudal position but also connectivity modules selective for fin/limb versus axial

muscle control (Alexander et al. 2009, Catela et al. 2015, Jung et al. 2018, Krumlauf 1994). This rostrocaudal patterning provides a genetic substrate for the diversification of neuron subclasses, circuit composition, and connectivity along the longitudinal axis of the spinal cord.

All cardinal classes span the rostrocaudal extent of the spinal cord, but there is significant variation in organization and distribution along this axis. Immunostaining in embryonic mouse spinal cord showed that the rostrocaudal distribution of different cardinal classes varies (Francius et al. 2013). While the V3 class was homogeneously distributed, V1 neurons exhibited differential distribution of subtypes along the rostrocaudal axis. V0 and V2 populations exhibited changes in mediolateral and dorsoventral positioning between brachial, thoracic, and lumbar segments. More sensitive assays have recently revealed variations in subpopulations along the rostrocaudal axis (Hayashi et al. 2018, Sweeney et al. 2018). Within V1 neurons, subsets defined by combinatorial expression of transcription factors were enriched at either the thoracic or lumbar level (Sweeney et al. 2018), aligning with hypaxial and limb motor nuclei at these segments. Even within thoracic segments, thoracic-enriched V1 subtypes showed distinct distributions, with some types biased to one subregion and others distributed uniformly. Collectively, these results suggest differences in spinal cord composition, connectivity, and function that may underlie the observed variations in rhythmogenic capacity along the rostrocaudal axis.

In contrast, in zebrafish, the V0, V1, and V2 classes are largely homogeneously distributed in the rostrocaudal axis (Björnfors & El Manira 2016, Callahan et al. 2019, Kimura et al. 2006, Menelaou et al. 2014, Picton et al. 2022, Sengupta et al. 2021). These results may suggest that segmental variations are more relevant to the functional demands of limb control, or they may reflect the larger locomotor repertoire of mice compared to fish. At the subtype level, however, in both mice and zebrafish, V2a neuron subclasses are differentially distributed in the longitudinal axis: V2a neurons with bifurcating axons sending collaterals to the brainstem are located in the rostral spinal cord, whereas V2a neurons with descending axons are biased toward the caudal spinal cord (Hayashi et al. 2018, Menelaou et al. 2014). These results suggest that some asymmetries in rostrocaudal organization are conserved and are essential features of vertebrate locomotion. In addition to these variations in subpopulation distribution, it remains largely unknown whether intrinsic cellular properties, such as input resistance or gain, vary systematically in this axis within a population, as in motor neurons and muscle fibers (Kernell et al. 1985). Collectively, these studies demonstrate variations in genetic and cellular distributions along the rostrocaudal axis, highlighting the need to consider regional identity in analysis.

4.2. Connectivity Along the Rostrocaudal Axis

A natural consequence of differential longitudinal distribution of neuronal subpopulations is circuit variations along this axis. Within an interneuron class, rostral and caudal populations exhibit differences in connectivity. For example, in CSF-cNs, rostral populations form synapses with cranial motor nuclei and hindbrain V2a neurons, whereas midbody and caudal CSF-cNs form synapses with axial motor neurons and V0v neurons (Wu et al. 2021). Superimposed on these segmental variations, spinal interneurons can also display an additional degree of complexity in connectivity along the rostrocaudal axis: local versus distal targeting. Subsets of interneurons project long axons in this axis. These neurons, which are termed propriospinal, are found in almost all cardinal classes, can be excitatory or inhibitory, and have been implicated in postural control and interlimb coordination (Al-Mosawie et al. 2007; Alstermark et al. 1987; Flynn et al. 2017; Juvin et al. 2005, 2012; Kostyuk & Vasilenko 1979; Laliberte et al. 2019; Pocratsky et al. 2020; Quinlan & Kiehn 2007; Ruder et al. 2016; Soffe et al. 2001). What are the long-range targets of propriospinal neurons?

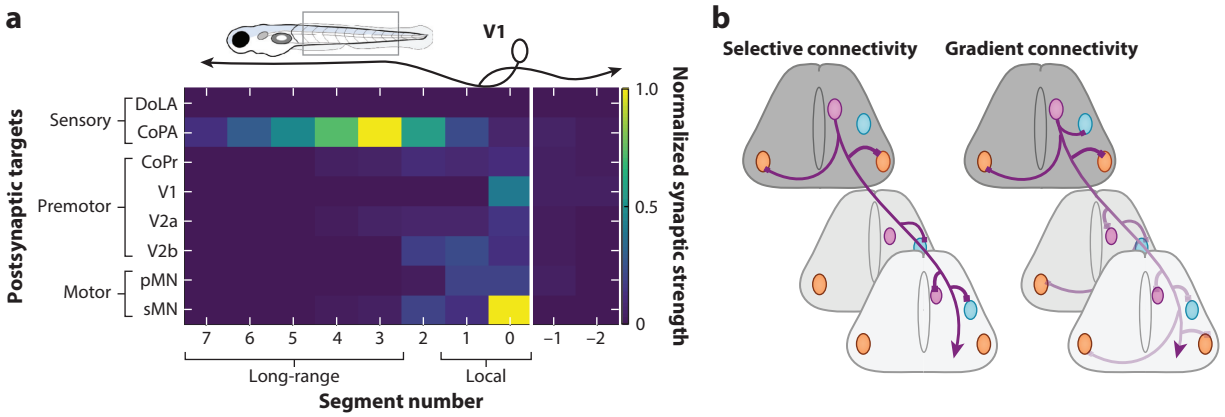

Figure 3

Differential connectivity in the rostrocaudal axis. (*a*) In larval zebrafish, V1 neurons form local synaptic connections with motor and premotor neurons and long-range connections with sensory targets. The heatmap shows connections from V1 neurons to eight sensory, premotor, and motor target populations (rows). Each square represents the median value of evoked charge transfer of that cell type in the respective rostrocaudal position (segments, *x* axis). All charge transfer values were normalized to cell conductances to allow comparison across cell types. Panel *a* adapted with permission from Sengupta et al. (2021). (*b*) Schematics for two models of long-range connectivity. (*Left*) Selective connectivity, in which a propriospinal neuron forms synaptic connections with different partners as its axon traverses the rostrocaudal axis. (*Right*) Gradient connectivity, in which a propriospinal neuron contacts similar types of neurons locally and long range but with a gradually decreasing number of connections at long distances. Abbreviations: CoPA, commissural primary ascending (likely homologous to dI5); CoPr, commissural premotor (likely dI6, V0); DoLA, dorsal longitudinal ascending (likely homologous to dI4); pMN, primary (fast) motor neuron; sMN, secondary (slow) motor neuron.

Anatomical tracing of cervical, descending interneurons shows that in many cases, their axons target regions outside the motor neuron lamina (Ruder et al. 2016), implying that interneurons preferentially target nonmotor neurons at long ranges. Consistent with this idea, transsynaptic tracing experiments suggest relatively sparse long-range interneuron connectivity to motor neurons (Levine et al. 2014, Ni et al. 2014, Ronzano et al. 2021). Systematic connectivity analysis of the V1 population in zebrafish showed that long-range connections fall almost exclusively onto sensory, not motor, neurons, whereas short-range connections occur with a wide range of motor and premotor partners (Sengupta et al. 2021) (**Figure 3*a***). This pattern has also been indirectly supported in mammalian Renshaw cells (Jankowska & Smith 1973). Similarly, ascending V3 neurons appear to preferentially target interneuron, not motor neuron, laminae (Zhang et al. 2022), suggesting that ascending connectivity may be particularly distinctive at local and long ranges. As a potential differentiator between local and long-range projections, a recent study using transcriptomic data in mice showed that short- and long-range projecting neurons form genetically separable groups, marked by the expression of *NeuroD2* and *Zfhx3*, respectively (Osseward et al. 2021). Long-range communication to sensory or nonmotor populations would provide efficient routes for rostrocaudal coordination (Juvin et al. 2012) and sensory gating (Knogler & Drapeau 2014).

Based on these observations, it is tempting to hypothesize that there may be a differential pattern of connectivity in the short versus long range in which local synaptic connections primarily target motor and premotor pools, whereas long-range communication occurs mostly between interneurons. An alternative hypothesis is simply that all interneuron connectivity is biased to local regions and that the subset of spinal neurons projecting over long distances connects equally well to motor and sensory targets (**Figure 3*b***). To distinguish between these ideas, the field will need more systematic longitudinal mapping of connectivity. This could take the form of optogenetic mapping of physiological connectivity (Sengupta & Bagnall 2022, Sengupta et al. 2021),

and anatomical mapping of propriospinal connectivity (Ruder et al. 2016). Improved labeling of interneuron subclasses will aid in both approaches. Excitatory connections may also be suitable for all-optical mapping approaches (Fan et al. 2020); subthreshold synaptic potentials are still difficult to assess with both calcium and voltage indicators, but recent improvements in transmitter sensors (Marvin et al. 2018, 2019) may overcome these limitations.

5. CONCLUSIONS AND FUTURE DIRECTIONS

While the spinal cord field has benefited tremendously from new genetic approaches for identifying distinct neuronal populations, it remains a struggle to integrate cellularly defined identities with circuit or function. The advantage of genetically rather than functionally defined populations is that they are more likely to be consistent across both laboratories and species, but at the moment there is a great deal of uncertainty regarding what truly constitutes a spinal interneuron population. These genetically defined classifications may obscure a more fundamental relationship between physiology and behavior in which movements are generated by pools of neurons organized by development or activity. Our understanding of spinal cord circuits for movement would benefit from community efforts toward defining and understanding interneuron classes in a systematic fashion.

FUTURE ISSUES

1. Can birthdate-associated variations explain the apparent continuum of genetic expression patterns in ventral horn interneurons?
2. Do early- and late-born spinal interneurons generally exhibit distinct physiological properties?
3. Does spinal neuron birthdate correlate with the speed of locomotor recruitment in species other than fish?
4. Are there patterns of connectivity among interneuron populations at the level of genetically identified subclasses?
5. Does interneuron connectivity systematically vary along the rostrocaudal axis or across local and long-range connections? Do ascending and descending connections follow similar or different logic?
6. Do genetic homologies across species reflect similar underlying contributions to circuit computations, or have neuron classes been co-opted into such different circuits that the results from one species are unlikely to be predictive in another?

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