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Peeling back the layers of locomotor control in the spinal cord

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Abstract

Vertebrate locomotion is executed by networks of neurons within the spinal cord. Here, we describe recent advances in our understanding of spinal locomotor control provided by work using optical and genetic approaches in mice and zebrafish. In particular, we highlight common observations that demonstrate simplification of limb and axial motor pool coordination by spinal network modularity, differences in the deployment of spinal modules at increasing speeds of locomotion, and functional hierarchies in the regulation of locomotor rhythm and pattern. We also discuss the promise of intersectional genetic strategies for better resolution of network components and connectivity, which should help us continue to close the gap between theory and function.

Introduction

"It is inessential at present whether the lumbar centres are two in number and situate on opposite sides of the spinal cord; or whether they are four in number and situated in antagonistic pairs on each side of the cord; or whether there are more than four in number."

T. Graham Brown, 1911 [1]

The evidence that networks of neurons within the spinal cord are sufficient to generate locomotion is over a century old. Although this idea remains largely uncontested [2], work since then has led to modifications of the original model Brown put forth to explain his observations, namely the 'half-center' hypothesis. According to this concept, locomotion relies on pools of premotor excitatory interneurons locked in rhythmic alternation by fatigable sources of inhibition. While Brown was understandably less concerned with the number, location or scalability of neuronal half-centers, more recent models have attempted to account for the complexity of motor coordination during locomotion, where muscles are

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not always purely antagonistic and movements are not always at the same speed. Here, we highlight recent studies testing some of the major predictions arising from past and current models. To this end, we will focus on work using optical and genetic approaches to interrogate the spinal locomotor networks of mice and zebrafish.

Spinal network modularity

In mammals, spinal motor neurons are grouped into functionally and spatially distinct pools according to the muscles they innervate [3]. For locomotion, these pools must be appropriately coordinated within and between two sets of bilaterally paired limbs (Fig. 1A). Given the prohibitive complexity of independently controlling motor neurons, theories about premotor control have also invoked a pooled or 'modular' organization, as exemplified by the 'unit burst generator' (UBG) hypothesis of Sten Grillner [4]. According to this idea, motor pools controlling flexor or extensor movements around different joints have their own dedicated UBG made up of interconnected excitatory interneurons, whose purpose is to drive rhythmic motor activity (Fig. 1B). The UBG concept deviates from the half-center hypothesis in that reciprocal inhibition is not a prerequisite for rhythmicity, which allows UBGs to change their relative state of coupling (e.g., antagonistic *versus* synergistic) and provides a basis for variations in limb coordination during locomotion.

A recent paper from the Kiehn lab has tested one of the major predictions of a UBG type organization, namely that motor pools should be able to generate rhythmic activity independently. To do so, Hagglund *et al.* [5] used transgenic lines of mice in which optogenetic actuators were selectively expressed in spinal glutamatergic neurons. The advantage over past work is that this approach allowed for the targeted and reversible activation and silencing of restricted regions of the spinal cord [6]. Using this method, combined with bulk recordings from ventral roots and more selective recordings from rootlets, the authors demonstrate the independent bursting capability of flexor- and extensor-related motor pools in both spatial distant and more closely apposed locations in the lumbar spinal cord.

So how might these independent UBGs be coordinated during locomotion? At least for movements within a limb, flexor-extensor alternation during locomotion has been attributed to the reciprocal actions of ipsilateral sources of inhibition [7]. A recent paper from the Goulding lab has examined the role of ipsilateral inhibitory interneurons in mediating flexor-extensor alternation in the hindlimbs. The work relied on methods to manipulate these populations based on their developmentally-derived molecular signatures [8], specifically En1-labeled V1 neurons from the p1 progenitor domain and Gata3-labeled V2b neurons from the p2 progenitor domain. Consistent with the UBG hypothesis, Zhang *et al.* [9] demonstrate that in the absence of ipsilateral inhibition flexor and extensor motor pools can burst rhythmically, however the pools become synchronized (Fig. 1B). The work also demonstrated a functional redundancy in that flexor-extensor alternation is only abolished after silencing both the V1 and V2b populations, suggesting that inhibitory flexor-extensor modules are found in both groups.

While the organization of separate UBGs for control of ipsilateral motor pools was considered a specialization of limb control, recent work has extended this concept to the axial networks controlling swimming in larval zebrafish. Using paired voltage-clamp recordings to compare the relative timing of excitatory and inhibitory synaptic currents during 'fictive' swimming, Bagnall and McLean [10] reveal that the inputs to motor neurons that innervate either dorsal or ventral trunk musculature along one side of the body are not completely shared. To link these observations to molecularly-defined spinal circuitry, the authors drove stochastic expression of a light-gated channel into a major source of ipsilateral premotor excitatory drive, namely Chx10-labeled V2a neurons [11–14]. Consistent with the assessments of network drive, optogenetic activation of sparsely labeled V2a neurons demonstrated the existence of mutually exclusive input patterns (Fig. 1C). Although the original formulation of UBGs suggested that left-right alternation represented the minimum functional module in primitive axial networks [4], there is also evidence for separate spinal drive to motor neurons innervating dorsal and ventral trunk muscles in lampreys [15,16]. Taken together, the findings suggest a finer scale modular organization of axial premotor networks than previously appreciated and also provide an early evolutionary template for independent control of musculature on the same side of the body, as proposed by the UBG concept (Fig. 1C).

Speed control

When considering the manifestation of spinal network modularity, it is important to remember that motor pools are not uniform in their composition, nor are they likely to participate equally in movements of varying speeds and strengths [17]. In this sense, the UBG hypothesis needs to be integrated with another prevailing concept, namely Elwood Henneman's 'size principle' [18]. Stronger movements are associated with the recruitment of motor neurons that drive muscle fibers with more force output. These motor neurons tend to be larger and less excitable and so presumably require more drive to get them to threshold (Fig. 2A). A recent study using voltage-clamp recordings in larval zebrafish has begun to elucidate the synaptic basis for synchronizing activity among heterogeneous motor neurons during rhythmic locomotion [19]. In particular, increases in the frequency of fictive swimming are associated with a preferential increase in excitatory drive to less excitable motor neurons (Fig. 2A). This observation favors arguments for network properties determining the coordinated recruitment of motor pools [20], as opposed to the exclusive contribution of intrinsic membrane properties, as predicted by the size principle [21].

To this end, recent work in zebrafish is beginning to reveal the sources of differential drive to motor neurons during increases in swimming frequency (Fig. 2B). At larval stages, premotor V2a neurons vary in their participation in swimming based on their spatial location; at the highest frequencies subsets of ventrally located V2a neurons are inhibited as more dorsal ones are engaged [12,14,22,23]. A recent anatomical study in larvae has demonstrated that more dorsal V2a neurons have the potential to make systematically more connections to less-excitable motor neurons by virtue of convergent intersegmental axonal projections [24]. This could provide the observed bias in rhythmic excitation during faster swimming [19]. In juvenile/adult zebrafish young enough to use Chx10 expression to identify V2a neurons [25], the spatial recruitment pattern is obscured by neuronal migration,

but there are still differences in V2a activation patterns from low to medium frequencies of swimming evoked by bulk electrical stimulation [26]. A recent paper from the El Manira lab has used recruitment over this range of frequencies in older zebrafish to reveal functional patterns of premotor V2a connectivity. Using paired patch-clamp recordings, Ampatzis *et al.* [27] find that V2a neurons and motor neurons recruited around the same frequency are biased in their local connectivity to one another. Collectively, the work suggests that increases in speed are accompanied by the incorporation of V2a circuits that can drive the appropriate motor neurons (Fig. 2B).

In mammals, ground speed is not only a function of the level and frequency of motor pool activation during cyclical footfalls, but also a product of changes in inter-limb coordination (Fig. 1A). One example of a speed-dependent difference in inter-limb coupling in mice, as in many other mammals [4], is the transition from left-right alternation of the hindlimbs at slower speeds to left-right synchrony at the fastest speeds [28]. A recent paper from the Kiehn lab has revealed a functional dichotomy within the Dbx1-labeled V0 population during the maintenance of left-right hindlimb alternation at different speeds of locomotion. Most V0 neurons are commissural, and are thus well suited to regulate left-right limb coordination, but they are divided into inhibitory ($V0_D$) and excitatory ($V0_V$) subsets based on their spatial location and respective expression of the transcription factor, Pax7 [29–31]. Talpalar *et al.* [31] took advantage of the latter difference to selectively remove crossed inhibition or crossed excitation, and demonstrated that inhibitory $V0_D$ neurons are essential for maintaining left/right alternation at slow speeds, but are dispensable at faster speeds, when excitatory $V0_V$ cells appear to play a more prominent role (Fig. 2C). Specifically, in the absence of $V0_V$ cells, mice make an earlier transition to a near synchronous hopping gait than they normally would. This study adds to the growing body of evidence suggesting that in mice, as originally reported in zebrafish [22], there are different spinal circuit configurations driving increasing speeds of locomotion [32–36].

Separating rhythm from pattern

The UBG model presents the modular organization of locomotion as a single layer, where last-order interneurons (*i.e.*, those driving motor neurons) also serve as first-order interneurons (*i.e.*, those driving other interneurons [37]). However, in a more recent model David McCrea and Ilya Rybak argue for a multi-layer organization, in which 'pattern' forming interneurons with both last- and first-order function are driven by 'rhythm' generating interneurons that are purely first-order [38]. The multi-layered concept arose to explain the occurrence of spontaneous 'deletions' in motor output, when premotor synaptic drive would completely drop out [39,40]. In some cases, deletions had no impact on the timing of subsequent motor bursts ('non-resetting deletions'), while in others the deletions were associated with a change in burst timing ('resetting deletions'). In particular, non-resetting deletions are difficult to reconcile with a single layer UBG model, if the same neurons responsible for recruiting motor neurons also control the timing of their activity [41].

Recent work from the Harris-Warrick and Gosgnach labs has begun to look for evidence of multi-layer control in mice, by monitoring the activity patterns of spinal interneurons during

non-resetting deletions. In V2a [42] and commissural dI6 [43] populations, as well as other unidentified interneurons [44], subsets fall silent during deletions, while others remain rhythmically active (Fig. 3A). These observations are consistent with the idea that neurons are embedded within either the pattern forming or rhythm generating layers, respectively. Another recent paper from the Kiehn lab has used an intersectional genetic approach to tease apart the control of rhythm *versus* pattern. Dougherty *et al.* [45] identify a novel population of ipsilaterally projecting excitatory interneurons marked by the expression of Shox2, which partially overlaps with the Chx10-labeled V2a population (Fig. 3B). Optogenetic or synaptic silencing of all Shox2$^+$ interneurons (red and purple in Fig. 3B) prevents the generation of faster locomotor-related rhythms, while leaving the patterning of flexor-extensor and left-right alternation intact. In this case, the selective impact on frequency was used to define a rhythm generating function. Critically, ablation of Shox2$^+$/Chx10$^+$ V2a neurons (purple in Fig. 3B) has no dramatic effect on frequency (*i.e.*, rhythm) or pattern. This makes it likely that the Shox2$^+$ non-V2a neurons (red in Fig. 3B) are responsible for the observed frequency perturbation, and that they, along with other excitatory interneurons, comprise the main rhythm generators in the rhythm generating layer.

The utility of a multi-layer organization is also bolstered by its ability to explain disruptions induced by genetic reorganization of spinal networks [46,47]. However, exploratory simulations based on UBGs also capture the major features of intra-and inter-limb coordination during locomotion [48]. Thus, the likelihood of a hierarchical UBG *versus* a multi-layered scheme is still debated [49–51]. More detailed maps of connectivity should help resolve this issue, by testing the last- *versus* first-order predictions of one concept over the other.

It is presently unclear whether axial networks would require a similar segregation of rhythm *versus* pattern elements. However, a recent paper from the Masino lab has demonstrated a functional dissociation in the control of the episodic structure and fine burst timing of motor activity during drug-evoked fictive swimming [52], leaving open the possibility that a multi-layer organization is also present in larval zebrafish.

Future directions

As the work described here illustrates, the investigation of spinal locomotor control has a long tradition of developing models to account for new experimental observations. Moving forward, predictions arising from increasingly detailed, biologically inspired computational models will need to be tested using methods that allow more precise control of circuit elements in behaving animals. The current state of the art uses genes identifying common early developmental origins, however there is a growing appreciation that even within progenitor domains there is considerable heterogeneity. For instance, recent work has demonstrated that time of differentiation plays a crucial role in generating anatomical and functional diversity within progenitor zones in mice [53–55] and zebrafish [56]. Consequently, identification of genes specific to neurons arising at different stages of development or better temporal control of inducible gene expression should facilitate a more targeted optogenetic assessment of circuit function.

Another important consideration relates to the distributed nature of spinal locomotor control. A recent paper has characterized the distribution of genetically identified populations along the length of spinal cord in mice [57], which reveals regional specializations resembling the columnar organization of motor pools controlling the limbs and trunk [58]. In addition, viral tracing methods have recently identified sources of premotor inputs to hindlimb motor neurons, which include long range descending V2a neurons [59] and local subsets of dl6, V0, V1, and V2 interneurons that also synapse with axial motor neurons [60]. Collectively, the work is beginning to outline molecularly-defined candidate neurons and patterns of connectivity responsible for coordinating movements of the hindlimbs with those of the forelimbs and trunk. However, it also raises important caveats for future work, namely that spinal premotor drive to lumbar motor pools is unlikely to come solely from local sources, nor are local lumbar circuits likely to be dedicated only to hindlimb movements.

A basis for the spatial and temporal integration of molecularly-defined appendage and axial circuits will also be provided by studies of metamorphosis in *Xenopus* frogs [61], in which optogenetics are now feasible [62], and can build on decades of work studying axial control [63]. Future optogenetic studies in zebrafish will also help in this endeavor. A recent paper from the Hale lab has demonstrated that slow swimming in larvae is associated with coordinated activation of pectoral fin and axial motor neurons, while at faster speeds the axial motor neurons take over as tonic activation of 'flexor' pools pins both pectoral fins against the body [64]. It will be interesting to see if such 'gait' transitions reflect dedicated *versus* shared limb/axial circuitry, whether hierarchical relationships between the two exist in speed-dependent manner, and if the patterns revealed for pectoral fin control continue on as caudal, anal, dorsal, and pelvic fins are sequentially added during zebrafish development.

Finally, if principles of spinal locomotor control are to be defined, it should be based on the breadth of their explanatory power. We have covered a few examples that appear to be conserved in mammals and fish. However, specializations related to different locomotor strategies are also informative, which will be evident not only between species, but also within them during development. In this sense, examinations of species- and age-dependent differences will need to account for differences in methodology, which often comes down to comparisons between studies performed either *in vivo* or *in vitro*. Continued advances in intersectional genetic and optical approaches that allow us to mark and monitor neurons into adulthood should help level the playing field, and as the techniques improve so too will our ability to distinguish what is inessential at present from what is essential in future.

Acknowledgements

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* of special interest

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projecting interneurons that overlaps with the V2a population. Shox2 neurons are rhythmically active during locomotion and subpopulations connect to commissural neurons, motor neurons, and other Shox2 neurons. The functional roles of Shox2 neurons are dissected using combinatorial genetics. When Shox2 neurons are synaptically silenced, the locomotor frequency decreases. However, ablation of Shox2 V2a neurons has no effect on locomotor rhythm or left-right alternation, demonstrating that the silencing of the Shox2 non-V2a neurons is responsible for the observed frequency reduction. Assuming the loss of rhythm generating neurons reduces locomotor frequency, the results place Shox2 non-V2a neurons as components of the hindlimb rhythm generator for locomotion.

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transsynaptic virus labeling to differentiate trunk and limb premotor networks. The trunk circuits are predominantly contralateral to the motor neurons. The limb circuits, on the other hand, are mostly ipsilateral and the dominance of ipsilateral over contralateral premotor neurons increases as the muscle innervated becomes more distal. Although the laterality of inputs may be distinct, neuronal classes involved in the coordination of hindlimbs during locomotion (dI6, V0, V1, and V2) are premotor to both limb and axial motor pools. The work has important implications for the relative binding of different axial and limb network modules during increases in locomotor speed.

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Highlights

- Limb and axial motor pool coordination are simplified by spinal network modularity

- Different spinal modules are deployed at increasing speeds of locomotion

- Hierarchical networks control the rhythm and pattern of locomotor activity

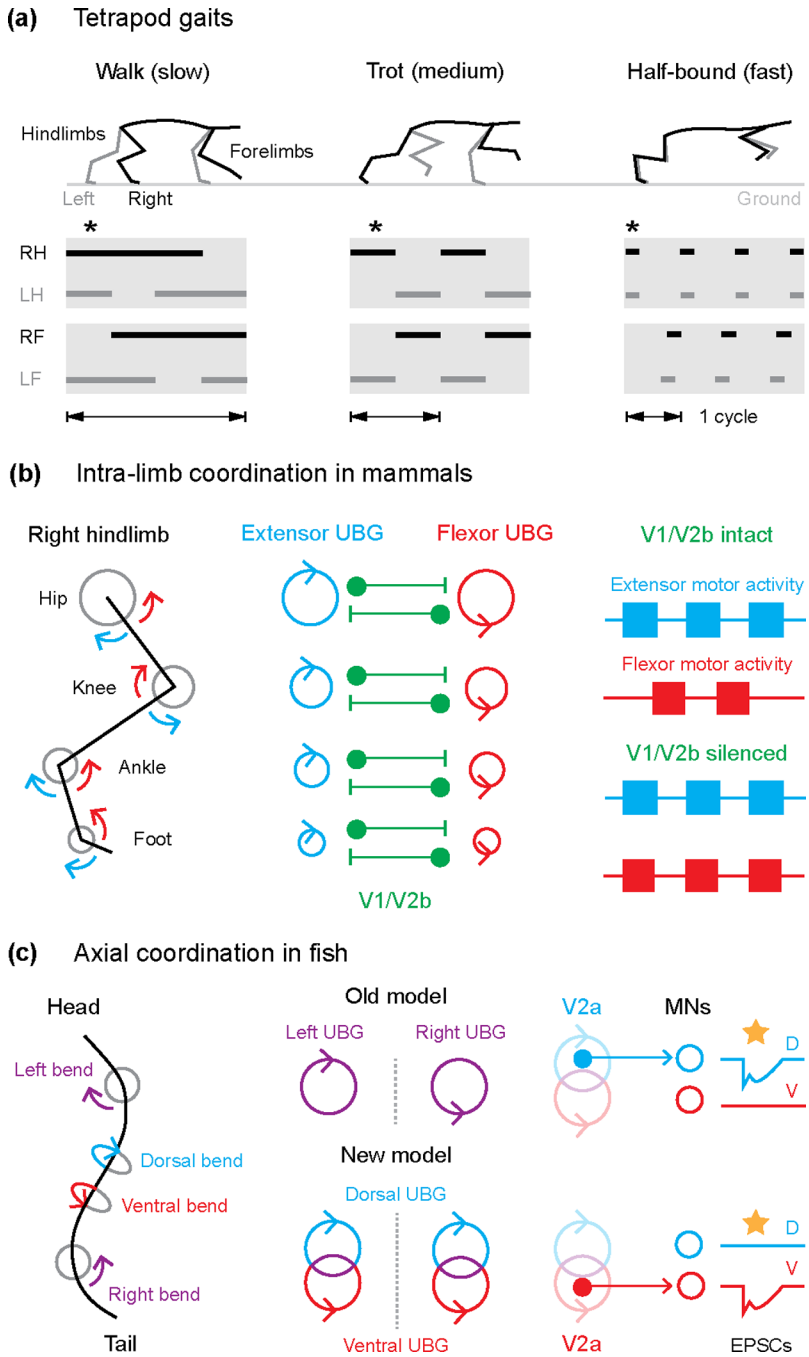- Intersectional genetics promises better resolution of components and connectivity

(a) Tetrapod gaits

(b) Intra-limb coordination in mammals

(c) Axial coordination in fish

Figure 1.
Modular control of ipsilateral motor pools in mammals and fish. **(a)** Changes in frequency and coordination between limbs associated with tetrapod locomotion are depicted by kinematic snapshots (upper panels) showing right (R, black) and left (L, grey) hindlimbs (H) and forelimbs (F) during three different gaits. Ground contact for each limb (bottom) is indicated by solid bars and asterisks indicate time points shown in the top panels. **(b)** Articulations around the joints within a limb (left) are divided into extensor (blue arrows) and flexor (red arrows) movements. Intra-limb coordination based on the unit burst

generator (UBG) hypothesis (adapted from [4]), likely involving the V1 and V2b ipsilateral inhibitory neurons, is illustrated in the center. Right panel summarizes results testing the involvement of V1/V2b interneurons in flexor-extensor coordination [9]. **(c)** Top down view of the midline of a fish swimming is presented on the left, where movements are coordinated across (left-right) and along the same side (dorsal-ventral) of the body. In the middle, an older UBG model is compared with an updated one, incorporating independent UBG control of dorsal (blue) and ventral (red) flexors along the same side of the body. Experiments demonstrating the segregation of V2a neurons into dorsal and ventral microcircuits are illustrated on the right [10]. Yellow stars indicate optogenetic activation of V2a neurons, which evokes electrical/chemical excitatory post-synaptic currents (EPSCs) in either dorsally projecting (D) or ventrally projecting (V) motor neurons (MNs).
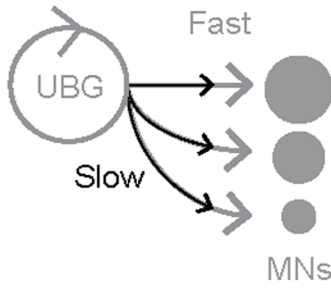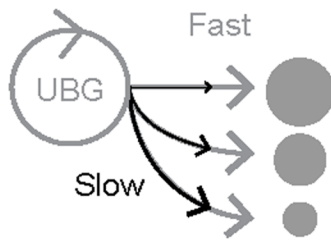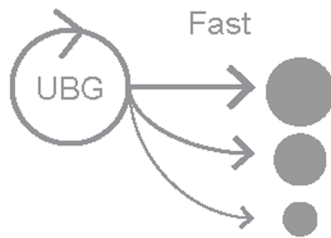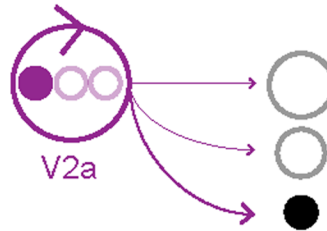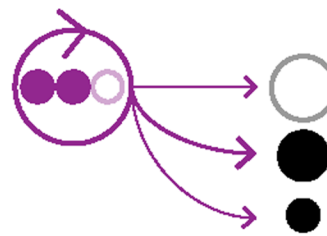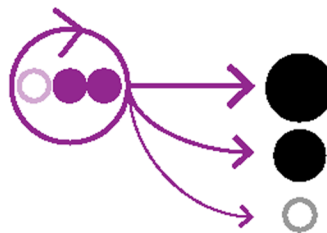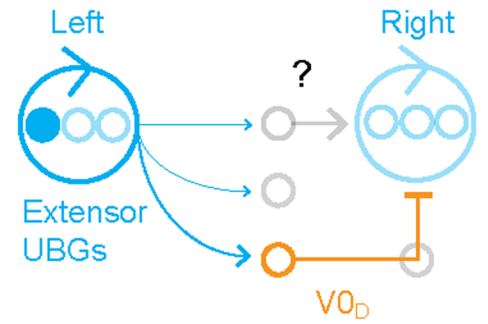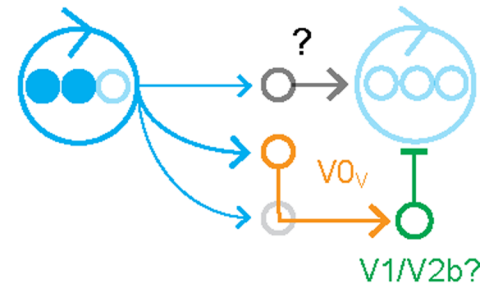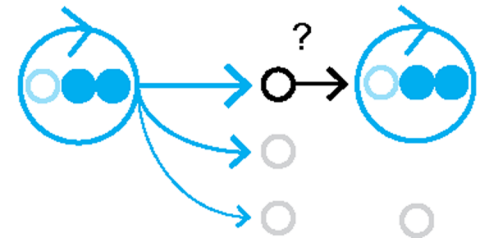
Figure 2.

Speed-dependent premotor control of motor neuron recruitment. **(a)** Incorporating the unit burst generator (UBG) hypothesis into models of motor neuron recruitment. According to the size principle (top), motor neurons (MNs) receive evenly distributed inputs, which are weaker at slow speeds (black arrows) and stronger at fast speeds (grey arrows), and orderly recruitment is a function of intrinsic excitability related to soma size [21]. In the network model (middle) [20], motor neurons recruited first receive biased drive (black arrows), and inputs biased to motor neurons recruited at faster speeds are added to the total excitatory drive (grey arrows). Critically, both models predict the same maximal distribution of excitatory drive across the motor pool at the fastest speeds. A new model (bottom) based on [19] has maximal drive (grey arrows) weighted to neuronal excitability across the motor pool. **(b)** In larval and juvenile zebrafish, V2a neurons comprise the UBG and provide appropriately biased drive to the motor pool [24,27]. V2a neurons recruited at slow speeds (top, filled purple circles) are biased in their connectivity to motor neurons active at those

speeds (filled black circles). With progressively faster swimming, V2a neurons are recruited to drive the appropriate motor neurons (middle and bottom) and some V2a neurons and motor neurons fall silent (bottom, open circles) at the fastest speeds [22]. **(c)** Speed-related changes in left-right alternation circuitry in the mouse [31]. Left-right alternation is secured by inhibitory $V0_D$ and excitatory $V0_V$ commissural inhibitory neurons at slow (top) and medium (middle) speeds, respectively. Note the weak activation of excitatory commissural neurons at medium speeds, accounting for synchrony following $V0_V$ ablation. At the fastest locomotor speeds (bottom), synchrony between left and right sides is mediated by a currently unknown excitatory commissural connection.
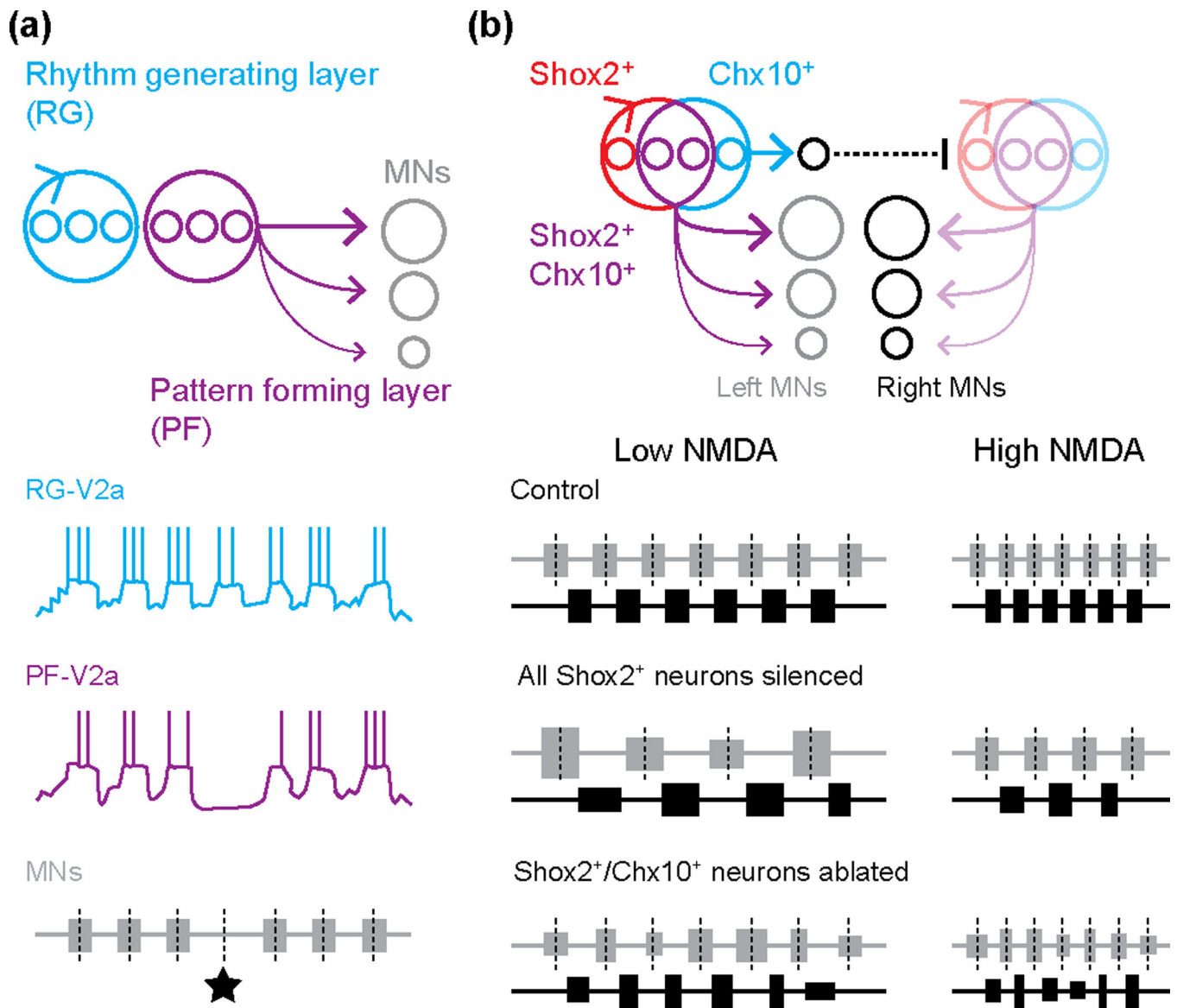
Figure 3.

Separate control of locomotor rhythm and pattern. **(a)** Top panel illustrates the functional separation of spinal neurons into first-order rhythm generating (RG, blue) and last-order pattern forming (PF, purple) layers. Below are representations of whole-cell patch clamp recordings from V2a neurons within each layer and motor neuron activity [42] during a 'non-resetting' deletion (black star). **(b)** Top panel illustrates the functional separation of neurons based on Shox2 and Chx10 expression. Shox2^{+} non-V2a neurons (red) are the rhythm generating neurons in the RG layer, while Chx10^{+} V2a neurons (blue) are also in the RG layer, but serve to control left-right alternation between rhythm generating neurons at certain speeds (by an as yet unidentified commissural pathway). Shox2^{+}/Chx10^{+} V2a neurons are in the PF layer (purple), connecting to either extensor or flexor motor neurons (MNs). Subtraction experiments (bottom) demonstrate the contribution of Shox2^{+} non-V2a neurons (red) to rhythm generation [45]. When all Shox2^{+} neurons (red and purple) are

optogenetically silenced, slower rhythms with more variable burst durations and cycle times are evoked by the same drug concentrations as compared to controls, but alternating patterns persist. When Shox2$^+$/Chx10$^+$ V2a neurons are ablated (purple), burst durations and cycle times are more variable, however the frequency of the rhythm is unaffected (black dashed lines).