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The functional diversity of spinal interneurons and locomotor control

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Spinal interneuron (IN) circuits ensure coordinated spatiotemporal muscle contractions necessary for complex locomotor behaviors. The identification of molecularly distinct spinal IN populations has enabled detailed and effective investigations into the organization of these circuits. Recent revelations of vast spinal IN diversity, particularly within cardinal spinal IN classes, have given rise to enormous challenges, as well as opportunities, in furthering our understanding of the functional circuit architecture underlying motor control. In the current review, we focus on recent studies revealing distinct spinal IN subpopulations assembled within rhythm-generating and pattern-forming spinal circuits. We briefly summarize how both general and task-specific spinal IN circuit outputs functionally combine, empowering a wide repertoire of locomotor behaviours.

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Introduction

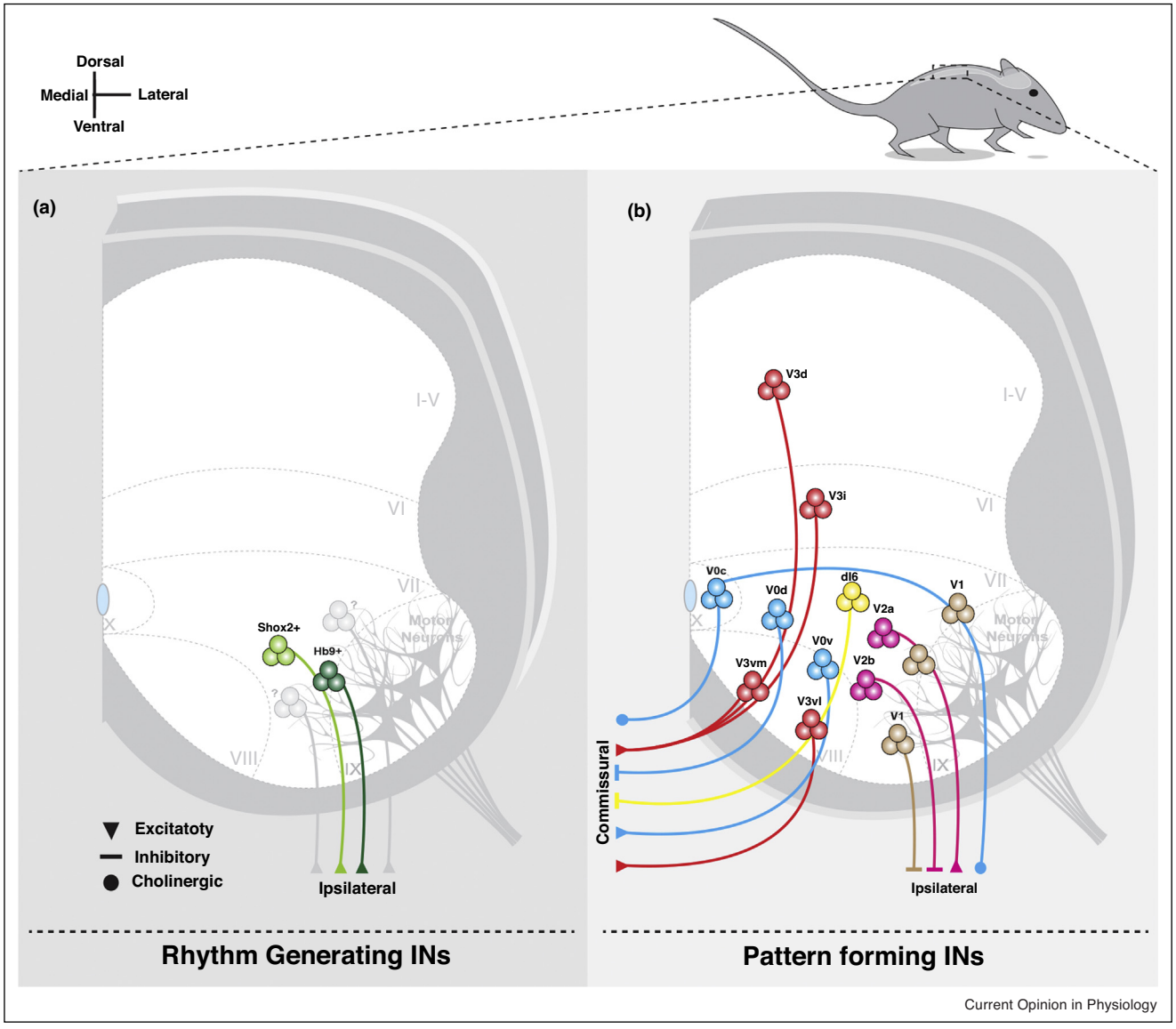
Locomotor movement has enabled vertebrate species to both survive and thrive for millions of years by empowering navigation through dynamic environments across land, sea, and air. Spinal interneurons (INs) form the basic motor circuits that ensure coordinated spatiotemporal muscle contractions. Distinct IN populations organize into functionally hierarchical modular circuits producing complex locomotor schemes – such as chewing, scratching, swimming, and walking [1–3]. In particular, the ventral side of the spinal cord functions as a central pattern generator (CPG) system [4], which can generate basic rhythmic and patterned motor outputs in the absence of descending supraspinal commands and ascending sensory inputs [5–10]. Furthermore, both experimental and computational studies indicate that locomotor rhythm and patterns can be generated, manipulated, and deleted independent of one another [7,11•,12–17,18•,19], suggesting a separation of rhythm-generating and pattern-forming spinal IN networks. As such, coordinated, flexible, and purposeful movements are direct products of IN diversity and the corresponding circuit logics within the spinal cord.

Currently, the most common and successful classification of spinal INs is based on their genetic identities. During early embryonic development, molecularly discrete progenitor domains organized along the dorsoventral axis of the folding neural tube give rise to 10 postmitotic cardinal IN populations (ventral: V0–V3, dorsal: dI1–dI6) [20]. Each cardinal group is defined by specific transcription factor expression profiles, distinctive anatomical properties, and general functional roles in locomotor control [2]. Yet, it has recently become clear that within each cardinal population substantial subpopulation heterogeneity exists, revealing a spinal IN complexity previously unappreciated. Multiple cardinal IN subpopulations have been delineated by their molecular, anatomical, physiological and functional properties. Furthermore, some of these functionally distinct subpopulations display task-specific involvements in motor control. This short review will serve as a snapshot of select recent studies addressing how diverse spinal IN subpopulations functionally integrate within rhythm-generating and pattern-forming spinal circuit layers (Figure 1), enabling a vast repertoire of locomotor behaviors.

Locomotor rhythm shared across IN types

Work utilizing fictive locomotor preparations have implicated ipsilateral glutamatergic spinal INs as both sufficient and necessary for rhythmic locomotor output [1,21,22]. Yet to date, only two molecularly defined spinal IN subpopulations have been suggested as direct contributors to rhythm generation. Firstly, glutamatergic Shox2$^+$ non-V2a INs innervate ipsilateral INs, but not motor neurons (MNs), and are rhythmically active during fictive locomotion [23]. When their synaptic transmission is blocked, spinal cords display significantly reduced locomotor frequencies without affecting left–right or flexor–extensor patterns [23]. However, spinal cords are still able to produce rhythmic outputs, implying that other IN populations are also important for rhythm generation. Indeed, a second glutamatergic and ipsilaterally projecting HB9$^+$ IN population has been shown important, though again not completely necessary, for increasing locomotor frequency [24].

Figure 1



Interneuron (IN) subsets in the ventral spinal cord organize into rhythm-generating and pattern-forming circuits.
Molecularly distinct ventral IN subpopulations possess-specific neurochemical (excitatory, inhibitory, or cholinergic) and anatomical (commissural or ipsilateral axon projections) properties. Distinct IN subpopulations then functionally integrate within rhythm-generating **(a)** or pattern-forming **(b)** locomotor circuit layers. [V0d (dorsal), V0v (ventral), V3d (dorsal), V3i (intermediate), V3vm (ventromedial), V3vl (ventrolateral)].

In addition, we would like to point out that genetic deletion of inhibitory dI6 [25] and V1 INs [26] and excitatory commissural V3 INs [27] also affects rhythmicity and frequency of locomotor output. Although it is still not clear if any of these INs are rhythm-generating cells or just regulate rhythm-generating circuits. Together, these results indicate rhythm generation and regulation are likely not confined to a single IN population but shared across several (Figure 1a, for a detailed review, please see the article in this same issue from Dougherty and Ha).

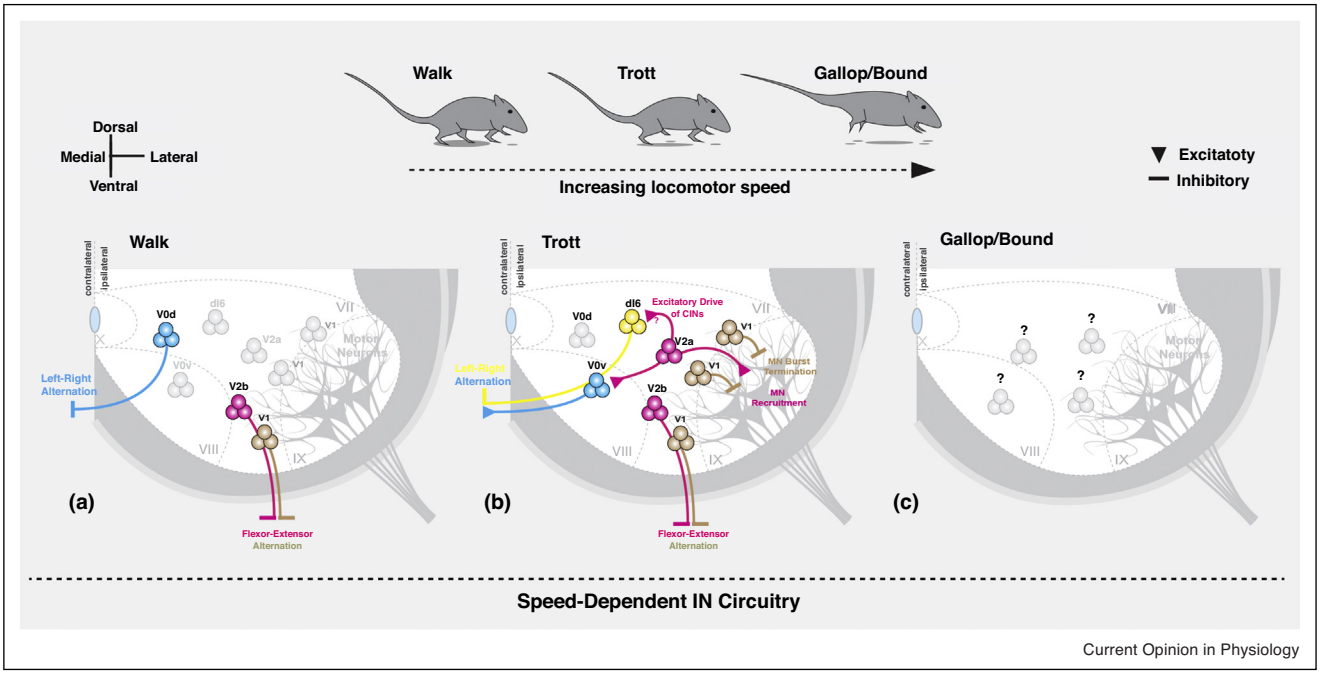
Locomotor pattern formation

Left–right coordination

Left–right alternation across different speeds

The spinal cord's ability to produce coordinated muscle contractions on either side of the body is paramount for successful locomotion. As an animal transitions between different locomotor speeds, it must accommodate changes in left–right contraction patterns to achieve optimal gaits (in the case of limbed animals) or swimming

Figure 2



Distinct pattern-forming IN circuits drive speed-dependent locomotor gait changes in mice.
At low locomotor speeds V2b and V1 INs collectively secure flexor–extensor alternations while V0d INs secure left–right hindlimb alternations producing a walking gait **(a)**. As locomotion increases to an intermediate speed, V2b and V1 INs continue to secure flexor–extensor alternations, V2a INs drive commissural INs and motor neuron recruitment levels, and dI6 and V0v INs secure left–right alternations, producing a trotting gait **(b)**. At maximum locomotor speeds the spinal IN circuits driving intermittent galloping/bounding gaits are unknown **(c)**.

frequencies (in the case of aquatic non-limbed animals). During limbed locomotion, wildtype animals exhibit distinct locomotor gait phenotypes with increasing speeds [28,29•]. As mice increase in locomotor speed they switch from an initial walking gait to a trotting gait, to finally a galloping/bounding gait. During both walking and trotting mouse hindlimbs alternate, while during galloping/bounding their hindlimbs switch to synchronous outputs. Interestingly, emerging evidence has begun to indicate distinct spinal IN circuit modules comprises dI6, V0, and V2a IN subpopulations that display speed-dependent left–right coordination recruitments (Figure 2).

V0 INs are mostly commissural INs originating from the Dbx1$^+$ p0 progenitor domain that project to contralateral motor neurons and ventral INs. Before becoming postmitotic, p0 progenitor cells separate into either excitatory V0 ventral (v) or inhibitory V0 dorsal (d) cells [30,31]. Although deletion of V0 INs (V0v & V0d) abolishes left–right alternation at all speeds, when V0v INs are exclusively ablated, left–right alternation is lost at trotting speed, but not at lower speed walking, while V0d deletion leads to a lack of left–right alternation at low frequencies during fictive locomotion [28,32]. Thus, while V0v and V0d subpopulations both drive left–right alternation, they functionally separate into distinct circuit modules that are differentially recruited across-specific locomotor speeds.

In addition to V0 INs, a portion of V2a INs has been implicated in coordinating left–right alternation at exclusively high locomotor speeds in mice. Chx10$^+$ V2a INs [33] form excitatory ipsilateral projections innervating MNs and other ventral IN populations. Interestingly, V2a ablated mice maintain normal left–right alternation at low locomotor speeds but switch to synchrony as locomotor speed increases [35]. V2a's recruitment is speed dependent, as the percentage of rhythmically bursting V2a INs significantly increases with increased locomotor frequency [36,37]. As V2a INs project significantly to commissural INs [35], some of which are V0v INs [34], it is possible that V2a and V0 INs form a high-speed alternating locomotor circuit module, whereby increased recruitment of ipsilateral V2a INs drives V0v INs enabling transitions from a walking to a trotting gait.

Similar to mice, work in the zebrafish has also revealed sequential V2a recruitment with increasing locomotor frequency, whereby the largest subset of recorded V2a INs are recruited at high locomotor frequencies [38]. Furthermore, V2a INs, like MNs, can be categorized into slow, intermediate, and fast subtypes-based on their

action potential firing properties, corresponding recruitment frequencies, and V2a-MN microcircuit connectivity patterns [38,39,40••]. Interestingly, in zebrafish, commissural glutamatergic V0v INs also display slow, intermediate, and fast recruitment subtypes [41]. Thus, incremental frequency-dependent MN recruitment, in addition to an MN's biophysical subtype properties [42], is likely further determined by upstream V2a and V0v modular microcircuit organizations.

Dorsal emerging dI6 INs have also been implicated in speed-dependent locomotor pattern formation. dI6 INs emerge from Lbx1$^+$ dorsal progenitor cells [43] and postmitotically form distinct subsets that express either WT1 and/or DMRT3 ([25]) as well as further undefined subset-specific molecular markers [44]. dI6 INs are mainly inhibitory [25] and are predominately commissural, although they form monosynaptic and disynaptic contacts with both contralateral and ipsilateral MNs [44]. While dI6 INs are dorsal originating, they settle into ventral and motor-related lamina by postnatal stages [25,44]. The majority of dI6 INs are rhythmically active during drug-induced fictive locomotion in the isolated mouse spinal cord [45,46•]. Additionally, electrophysiological recording of dI6 INs during fictive locomotor deletions have implicated dI6 IN subsets in both rhythm- generating and pattern-forming locomotor spinal circuits [44]. Functional or genetic deletion of either Wt1$^+$ [46•] or DMRT3$^+$ [25] INs in isolated neonatal spinal cords generates irregular fictive locomotor outputs with non-coherent left–right alternation. However, thus far, only DMRT3$^+$ dI6 IN functional outputs have been investigated, *in vivo* [25]. Dmrt3 mutated horses display difficulty transitioning from a trotting gait to a galloping gait with increasing locomotor speed. Instead, DMRT3 mutated horses express a 'pace' gait defined by synchronized movement of the legs on one side of the body at higher speeds. Thus, DMRT3$^+$ dI6 INs may play a critical role in proper locomotor pattern formation, particularly during high-speed locomotor tasks.

Balanced left–right activation

Currently, most studies of left–right coordination have focused on establishment of left–right alternation, but certain aspects of left–right activities have to be synchronized during locomotion. For example, most animals display a bounding gait at high speeds, which requires the synchronization of left–right limbs. In addition, to generate stable movement, the strength of the same muscle from left–right limbs as well as flexors–extensors on opposite sides of the body must be well matched in their output strength and patterns. Although it is still unclear what neuronal mechanisms underlie these left–right synchronizations, V3 INs have been shown to play some important roles in this function.

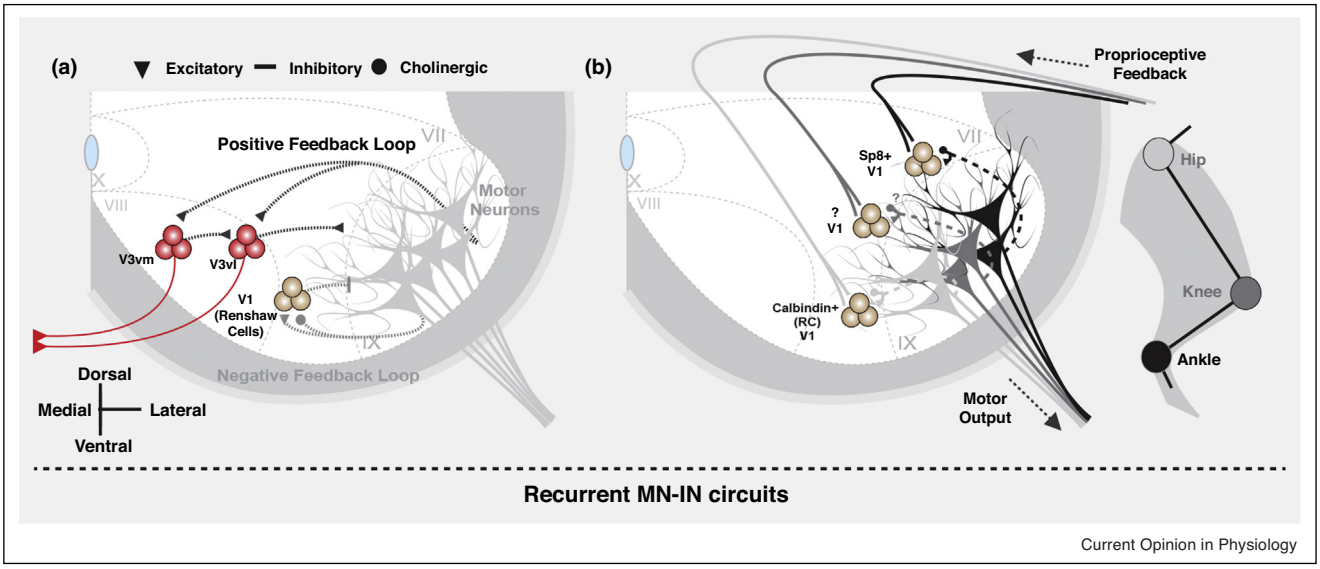
Sim1$^+$ V3 INs are glutamatergic and derived from the most ventral Nkx2.2-expressing p3 progenitor domain in the neural tube [27]. However, understanding V3 INs functional circuit logic has proven a challenge owing to their vast heterogeneity [27,61–63]. V3 INs diversify along the dorsoventral axis of the lumbar spinal cord separating into either dorsal (V3d) or ventral (V3v) subsets-based on their intrinsic membrane properties, axon projection profiles, and morphologies [61–63]. While V3d INs form exclusively commissural ascending propriospinal projections, V3v INs exhibit both commissural ascending and descending propriospinal axon projections [61,62]. Furthermore, although the majority of V3v INs (~85%) are commissural, select V3v INs also display ipsilateral projections [27,61], while some are likely bifurcating [64••].

To begin to delineate V3v local circuit logic, recent work by Chopek *et al.* [64••] utilized single-cell patch-clamp recordings in combination with holographic glutamate uncaging allowing for the mapping of local cell-cell V3 connectivity in the lumbar spinal cord. Chopek *et al.* [64••] revealed that contralaterally projecting V3v INs additionally form bilateral connections with local ipsilateral V3 IN and MN networks. Specifically, commissural V3 INs form a layered ipsilateral microcircuit in which medial V3v INs synapse onto larger lateral V3v INs, which then innervate local ipsilateral MNs. Ipsilateral MNs then also form excitatory glutamatergic synapses back onto both lateral V3v and medial V3v INs. Taken together, this work indicates that commissural V3v INs and local ipsilateral MNs form a positive feedback microcircuit (Figure 3a).

Whether the V3-MN layers of connectivity across the mediolateral spinal axis underscore functional layers of motor output is yet to be determined. However, the bilateral projections of V3v INs to both ipsilateral and contralateral spinal networks suggests that V3v INs may play important roles in securing coordinated and balanced excitation between distinct left–right flexor and extensor spinal circuits. Indeed, an early work by Zhang *et al.* [27] revealed that V3^{OFF} isolated spinal cord preparations display significantly uncoordinated and variable left–right and flexor–extensor ventral root bursting during fictive locomotion. Genetic deletion of V3 INs also led to unstable and imbalanced gaits. However, whether V3's role in securing stable excitatory coordination between left–right segments is a consequence of V3v's bilateral connectivities is yet to be determined. It also remains to be determined if V3 INs are required for left–right synchronization during maximum speed bounding gaits. Moving forward, V3 subpopulation molecular characterization is required to selectively target V3v INs and study their specific functions.

Thus, when considering the spinal IN networks coordinating movement across left and right sides of the body, it

Figure 3



Motor neurons (MNs) form distinct positive and negative feedback loops with ipsilateral and local IN populations.
Ventral V3(v) INs diversify into medial (V3vm) and lateral (V3vl) subpopulations and mediate recurrent positive feedback onto local MNs [64••]. V1 INs, initially described as Renshaw cells (RCs) [57], mediate recurrent negative feedback onto local MNs **(a)**. Dorsoventrally arranged V1 IN subsets receive joint-specific sensory and potentially motor neuron axon collateral inputs revealing potential joint-specific recurrent motor feedback circuits **(b)**.

is important to not only consider the IN networks regulating alternating or synchronous kinematics, but also the IN networks ensuring appropriate and robust left–right motor output kinetics.

Flexor–extensor alternations

The simultaneous activation and paired silencing of antagonist muscle groups around a joint is crucial for intralimb coordination and ultimately for motor execution. Both V1 and V2b INs form inhibitory synapses with ipsilateral INs and MNs, resulting in coordinated flexor–extensor alternations during locomotion and hindlimb reflex withdrawals [47]. V1 INs emerge from the p1 progenitor domain marked by the postmitotic expression of Engrailed-1 (En1) [20,48] while V2b INs emerge from Lhx3$^+$ progenitor cells and postmitotically express Gata2/3$^+$ [49]. When either V1 or V2b INs are independently silenced, mice maintain the ability to alternate flexor and extensor muscle contractions. However, the combined outputs of V1 and V2b are collectively necessary for flexor–extensor alternation [47], yet, they are differently biased towards flexor or extensor MN pools, respectively [50]. V1 INs preferentially innervate flexor MNs, whereby select V1 ablation results in hyperflexion during the swing phase. In contrast, V2b INs preferentially innervate extensor MNs, whereby select V2b ablation results in hyperextension during the stance phase [50]. Thus, while flexor–extensor alternation is functionally shared across V1 and V2b INs, their functional outputs are not redundant, as each IN class is functionally biased towards either flexor or extensor inhibition. Furthermore, it is possible that separate IN subpopulations within V1 and V2b classes additionally diversify in their joint-specific and/or MN pool-specific functions. Indeed, recent work by Bikoff *et al.* [51••] revealed multiple V1 IN subpopulations that receive distinct MN pool and muscle afferent inputs. Further work investigating the functional roles of these distinct V1 IN subpopulations in locomotion may help determine the significance of their diverse circuit organizations for flexor–extensor coordination.

Gain control of the motor outputs and reciprocal connection with motor neurons

MN outputs must be precisely controlled under different contexts to ensure appropriate muscle contraction strengths for a given motor task. Many spinal IN populations form monosynaptic connections with MNs, however, how they contribute to the regulation of the strength of MN outputs remains largely unknown. To date, V0c INs are the best-studied subpopulation involved in the gain control of motor output.

Within the V0v IN subclass, a subpopulation of V0c INs have been described to directly innervate MNs. V0c INs account for less than 5% of total V0 INs, they are marked by their embryonic and postnatal expression of Pitx2, and are cholinergic [52]. V0c INs form large synapses, called C-boutons, onto MNs, serving as a spinal cord intrinsic

neuromodulator system. V0c recruitment increases MN input-output gain through increased action potential firing frequencies [53]. V0c function was also been investigated *in vivo*. During swimming, mice require increased extensor bursting compared to over ground walking. However, V0c-deleted mice are unable to increase the MN output of ankle extensor gastrocnemius (GS) muscles, resulting in a prohibited swimming phenotype [52]. Thus, V0c INs regulate the input-output gains and resulting bursting amplitudes of appropriate MN pools required to achieve high-output motor tasks. Furthermore, V0c inputs may have different post-synaptic effects depending on the MN type they are innervating. In zebrafish, cholinergic spinal cord INs differentially regulate the excitability of biophysically distinct MN subsets [54].

MNs also receive vast excitatory glutamatergic inputs from spinal IN sources. For example, glutamatergic $Sim1^+$ V3 INs are a major class of excitatory spinal INs that directly innervate MNs [27]. MN pseudorabies virus injections revealed that a portion of V3v INs form monosynaptic inputs onto mainly commissural and some ipsilateral MNs [27]. Particularly, the recently revealed V3-MN microcircuit described in section 2a (Figure 3) [64••], further suggests that V3 INs can play important roles in controlling the activity of motor output across ipsilateral and contralateral spinal segments. Lastly, *in vivo* studies utilizing genetically-deleted V3 mice, show that, without V3 INs, animals are not able to increase the muscle strengths required for various locomotor tasks (unpublished data, y.z.).

Spinal IN Glyncinergic/GABAergic inputs to MNs may also play important roles in regulating the force of motor outputs, particularly, those IN subpopulations that form reciprocal connections with MNs. Renshaw cells were among the first spinal INs identified using traditional physiological and pharmacological methods. They have a unique reciprocal connection with MNs forming a recurrent inhibitory loop (Figure 3, [55–57,60]). Genetic studies have revealed that Renshaw cells are a subpopulation of early born V1 inhibitory INs [58,59]. Bikoff *et al.* [51••] revealed that dorsoventrally arranged V1 IN subsets (including Renshaw cells and other V1 subpopulations) receive joint-specific sensory and potentially MN collateral inputs. Thus, it is possible that distinct V1 IN subsets differentially integrate within joint-specific recurrent motor feedback circuits, though further functional studies are necessary to address these hypotheses (Figure 3b).

In addition to potential gain control of motor output, reciprocal connections between MNs and IN subpopulations can also provide a gateway for MNs to influence upstream spinal IN circuits, instead of merely being passive executive compartments of the spinal IN circuits. Song *et al.* [40••] have shown that subpopulations and V2a INs in zebrafish form gap-junctions with MNs, which are recruited under similar swimming frequency ranges. Interestingly, Falgairolle *et al.* [65•] also recently showed that, in the isolated mouse lumbar spinal cord, optical suppression or activation of MNs can significantly regulate the phase and frequency of drug-induced fictive locomotion. Together, these works provide evidence that MNs can influence spinal CPG networks in both non-limbed and limbed animals. Further studies are required to investigate the exact functions of these reciprocal connections.

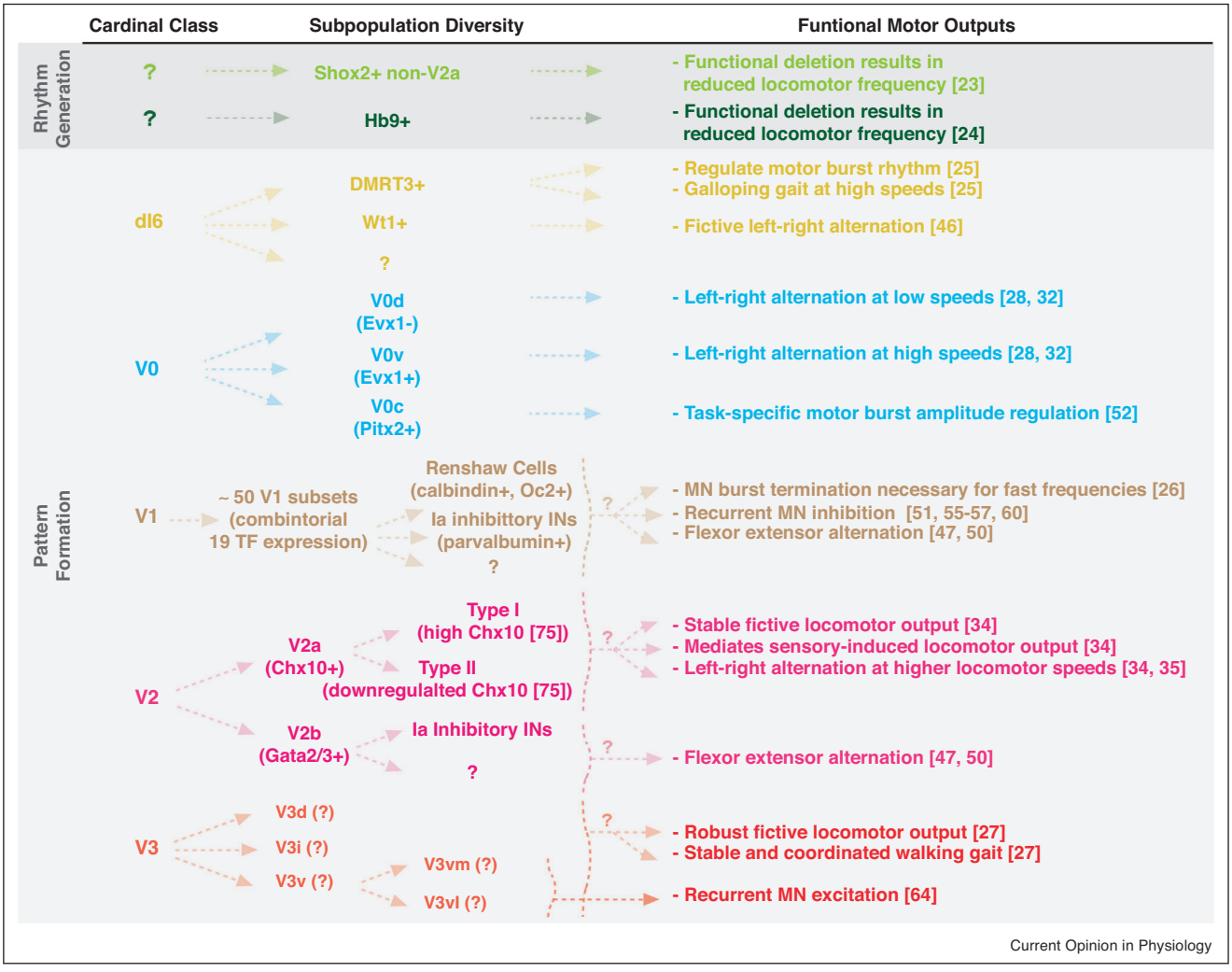
Taken together, extensive reciprocal connections between MNs and both local inhibitory and excitatory IN populations have recently been unveiled. These connections likely function as respective negative and positive feedback loops for MN activity during movement. Furthermore, MN outputs back onto spinal IN circuits suggests that MNs do not only integrate upstream spinal circuit activity but are active members of spinal CPG networks.

Concluding remarks

In the last two decades, tremendous progress has been made in uncovering the genetic and molecular diversity of spinal INs. Many works have led to enormous advancements in our understanding of the organization and functional logic of the spinal locomotor circuits, some of which were briefly summarized in this review (Figure 4). Such knowledge, however, also puts many challenges before us. It is still a daunting task to connect genetic and molecular identities of individual IN subpopulations to their physiological and functional phenotypes. Generating novel, or combining available, genetic, electrophysiological and optical tools to trace and manipulate the activities of individual subpopulations should remain a top priority. Furthermore, the potential task-specific recruitments of distinct IN subpopulations demand that functional investigations be undertaken across several motor control paradigms.

Lastly, we would like to emphasize that this review has mainly focused on ventral spinal INs that are involved in the 'core' CPG circuits that generate the basic rhythm and patterns of locomotion. Emerging studies have shown that subpopulations of ventral INs, as well as dorsal INs, integrate and mediate-specific sensory afferents [66–71] and descending supraspinal commands [72–74] shaping motor output. How all these inputs are integrated and ultimately translated into motor actions by spinal INs remains a fascinating question that has begun to be undertaken. Moving forward, we will need to combine all this knowledge to truly understand the mechanisms underlying movement.

Figure 4



Snapshot: spinal IN diversity and locomotor function I.

Conflict of interest statement

Nothing declared.

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Papers of particular interest, published within the period of review, have been highlighted as:

- • of special interest
- •• of outstanding interest

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The authors present a computation model of lumbar and cervical spinal interneuron circuits involved in speed-associated gait changes. In brief, their model demonstrated that long projecting cervical-lumbar V0d interneurons stabilize a low-speed walking gait, while a combination of both and V0v long projecting cervical-lumbar and local commissural interneurons stabilize a medium-speed trotting gait. These spinal circuits integrate external supraspinal and sensory inputs, which can induce gait changes independent of locomotor frequency.

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