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Master and Servant: Emerging Roles for Motor Neuron Subtypes in the Construction and Evolution of Locomotor Circuits

Jeremy S. Dasen¹

¹Neuroscience Institute, Department of Neuroscience and Physiology, NYU School of Medicine, New York, NY 10016, USA

Abstract

Execution of motor behaviors relies on the ability of circuits within the nervous system to engage functionally relevant subtypes of spinal motor neurons. While much attention has been given to the role of networks of spinal interneurons on setting the rhythm and pattern of output from locomotor circuits, recent studies suggest that motor neurons themselves can exert an instructive role in shaping the wiring and functional properties of locomotor networks. Alteration in the distribution of motor neuron subtypes also appears to have contributed to evolutionary transitions in the locomotor strategies used by land vertebrates. This review describes emerging evidence that motor neuron-derived cues can have a profound influence on the organization, wiring, and evolutionary diversification of locomotor circuits.

Introduction

Circuits embedded within the vertebrate spinal cord can generate motor output that reflects the rhythm and pattern of muscle activation deployed during basic locomotor behaviors. The wiring of spinal circuits relies on the ability of neurons to acquire specific subtype identities during development, which in turn establishes the initial architecture of connections within motor networks. Much of our understanding of the basic strategies used to generate neuronal cell types has emerged from elucidating the programs governing the subtype diversification of spinal motor neurons (MNs). Classic embryological manipulations, as well as more recent molecular characterization of MNs, have provided an in depth understanding of how a single neuronal class fractionates into hundreds of subtypes and establishes specific connections with postsynaptic targets.

Despite insights gained from resolving the genetic programs controlling the differentiation and peripheral connectivity of spinal MNs, the role of MNs in facilitating central connectivity within spinal circuits has been somewhat neglected. It has been assumed that MNs play passive roles as recipients of inputs provided by the activity of premotor

Correspondence: jeremy.dasen@nyumc.org.

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networks. Thus most attention has been placed on elucidating the origin and function of the various classes of interneurons (INs) that comprise central pattern generating networks.

Recent findings indicate that rather than acting merely as intermediaries between rhythm generating networks and muscle, MNs themselves can be instructive in establishing the connectivity and functional properties of locomotor circuits. Understanding the role of MN subtype identity in spinal circuit assembly may resolve how distinct motor behaviors are established and provide insights into the evolution of locomotor networks.

Establishing MN subtype diversity and peripheral target specificity

The intrinsic factors that contribute to the specification and diversification of MN subtypes, as well as the downstream effectors that facilitate the guidance of motor axons to their peripheral targets, are known with a high degree of cellular resolution [1-4]. MNs projecting to groups of related muscles, such as limb or axial muscle, are organized within motor columns generated at specific segmental levels of the spinal cord (Figure 1A). Within these columns MNs targeting individual muscles in the limb are clustered within motor pools, each pool occupying a stereotypic rostrocaudal and intrasegmental position.

Central to MN organization and peripheral connectivity are transcription factor-based networks acting shortly after progenitors exit the cell cycle. Spinal MN subtypes can be defined by the expression of specific combinations of transcription factors, including Hox proteins, Foxp1, Lim and Mnx-class homeodomain proteins, and a variety of motor pool-specific factors. Along the rostrocaudal axis, Hox transcription factors define the identity of MN columns generated at specific segmental levels [5] and act in concert with Lim HD proteins and Foxp1 to establish the diversity of MN pools. Most studies addressing how MN intrinsic networks determine synaptic specificity have focused on the guidance of motor axons to their peripheral muscle targets. MN subtype-restricted transcription factors contribute to axonal guidance and target selection by regulating the expression of surface receptors and ligands, including *ephrin/Eph* and *Ret/Gfra* family genes [4,6,7*].

The cell bodies of MNs are invariantly positioned within the spinal cord and form a central topographical map of peripheral innervation [6,8]. One feature of this topography involves the alignment of MN columnar subtypes relative to the position of their postsynaptic targets (Figure 1C). MNs within the lateral motor column (LMC) are generated at limb levels through the activation of the *Foxp1* gene by Hox transcription factors [9,10**]. At intervening thoracic levels, Hoxc9 represses the limb-level Hox network, and functions as a key determinant of LMC position relative to the limbs [10**].

Modulation in Hox-dependent programs appears to underlie evolutionary changes in MN distribution among vertebrates. In snakes, which evolved from limbed tetrapods, extension of *Hoxc9* expression into more rostral segments leads to a repression of the forelimb MN Hox network and a loss of LMC neurons at cervical levels [10**]. The absence of limb-innervating MN subtypes in snakes is also associated with a reversion from a limb-based locomotion to an axial-muscle driven undulatory strategy. While the precise basis for the

change in locomotor behaviors in snakes is unclear, *Hoxc9* may facilitate this transformation by repressing LMC specification or acting on multiple classes of spinal neurons.

Intrasegmental organization of MN pools

In addition to governing the organization of MN subtypes along the rostrocaudal axis, Hox factors specify the identity of pools targeting individual muscles. MN pools are clustered in invariant positions that are linked to the location of their peripheral limb muscle targets [11-13]. Pools located more dorsally within the LMC typically innervate distal muscles, while those positioned ventrally project to proximal targets [14]. Mutation in *Foxp1* leads to a loss of all molecular features of limb-innervating MNs, and the random selection of limb muscle by motor axons [15-17]. *Foxp1* and Hox proteins control MN pool organization, in part, through regulating type II cadherin expression. Mutation of *catenins*, intracellular mediators of cadherin signaling, disrupts the ability of LMC neurons to coalesce into motor pools [18]. Interestingly, despite the scrambled position of pools in *catenin* mutants, MNs still target muscles appropriate for their molecular identity, indicating MN clustering and muscle-specific connectivity are controlled through independent sets of Hox/*Foxp1* effectors. Because *catenin* mutations likely affect multiple intracellular signaling pathways, and MN disorganization has been reported only in *N-cadherin* mutants [18], the role of cadherins in MN clustering remains to be fully resolved.

While studies point to a role of cadherin signaling in the organization of motor pools, how the segregation of MN columnar subtypes is achieved is not well understood. An early step in columnar organization involves the separation of ventrally and dorsally projecting subtypes along the medio-lateral axis of the spinal cord. Medial motor column (MMC) neurons are positioned near the midline and project to dorsal epaxial muscles. All other MNs typically reside more laterally and initially pursue ventral trajectories. In mice mutant for *Foxp1*, *Hox* genes, or *catenins* the segregation of MMC and non-MMC neurons is preserved. A recent study shows that the clustering of MNs into columns relies on *Pbx* proteins [19**], transcription factors essential for high fidelity binding of Hox proteins to target sites [20]. Similar to *Foxp1* mutants, in the absence of *Pbx* genes all Hox-dependent programs in MNs are lost. Surprisingly, the remaining MMC and non-MMC neurons are intermixed, revealing *Pbx* genes are essential for the coalescence of motor columns (Figure 1B) [19**]. These studies also revealed an unanticipated role for *Pbx* genes in specifying MMC identity, a population thought to differentiate in a Hox-independent manner.

The organization of MNs into columnar and pool groups appears to be conserved amongst amniotes, but has not been established in other vertebrate species [21,22]. In zebrafish, MNs targeting dorsal and ventral axial muscle are largely intermixed [23,24]. Nevertheless, zebrafish MNs are functionally organized along the dorsoventral axis, where groups of MMC-like neurons are recruited at distinct locomotor speeds [25,26*]. This organizational feature may have evolved to coordinate activation of MNs that drive specific types of undulatory locomotion, such as slow swimming or escape reflexes. In contrast, MMC neurons in tetrapods are typically associated with postural stabilization, while locomotion is driven by LMC neurons. The organization of MNs in tetrapod may have appeared during the

transition of vertebrates to terrestrial habitats, or selectively lost in lineages adapted to undulatory locomotion.

Motor neuron position as a determinant of synaptic specificity in spinal circuits

What role does MN organization play in locomotor circuit connectivity? Studies on the wiring of spinal reflex circuits suggest that one function may be to facilitate the specificity of connections between proprioceptive sensory neurons (pSNs) and MNs. Mutations that affect motor pool differentiation, such as in the *Foxp1* gene, causes MNs that target limb muscle to occupy random positions within the spinal cord. Nevertheless, pSNs project to the same position within the spinal cord regardless of whether the MN receiving sensory input targets an inappropriate muscle peripherally [17]. These observations have led to a model in which the role of MN clustering and positioning is to establish the appropriate vicinity for where sensory-motor synapses can form (Figure 2A). Recent studies on the connections between pSNs and spinal interneurons reinforce the conclusion that target cell position may be a general mechanism governing connectivity within sensory-motor circuits [27,28*].

While control of MN settling position appears to be one strategy in determining sensory-motor specificity, transcriptionally controlled molecular recognition programs also have important roles. The transcription factor *Pea3* is expressed by a subset of limb-level motor pools and is required for multiple facets of differentiation, including MN clustering and intramuscular axonal branching [29,30]. *Pea3* is also essential to establish appropriate connections between pSNs and MNs, acting by determining the morphology of MNs dendrites and regulating expression of the repulsive guidance receptor ligand *Sema3e* [31-33]. Mutation in *Sema3e* does not affect MN position, but causes pSNs to target MN pools incorrectly (Figure 2A) [33]. These observations indicate that MN position alone is not a sole source of information for appropriate connectivity, and that MN-derived recognition cues are critical in sensory-motor reflex circuits. Sensory-motor connectivity therefore appears to involve an early MN-independent program, that dictates early pSN projections, and a MN-dependent program controlling fine-scale synaptic specificity.

Premotor interneurons and the establishment of locomotor behaviors

Vertebrate locomotion is facilitated by central pattern generating (CPG) circuits that are essential in coordinating MN firing across the left and right sides of the spinal cord (L-R CPG), and orchestrating reciprocal activation of limb extensor and flexor muscles (E-F CPG). Significant progress has been made in characterizing the classes of spinal INs that establish the rhythm and pattern of CPG output [34,35]. Left-right alternation relies on V0 INs which cross the midline and provide reciprocal inhibition of the contralateral CPG [36,37]. Coordination of extensor-flexor muscles requires V1 and V2b inhibitory INs [38,39*]. Several additional classes of molecularly defined spinal INs determine the robustness of CPG output as well as modulating MN firing patterns at different locomotor speeds [34].

While the identity and function of the core neuronal constituents of CPG circuits have been elucidated, how premotor networks engage specific MN populations is unclear. Because a single MN can receive inputs from a variety of presynaptic neuronal classes, it has been challenging to determine if there are rules that govern CPG-MN connectivity. One possible mechanism could be through generating INs of a similar diversity to that seen in MNs. Evidence in support of this idea has emerged from studies on the diversification of spinal IN subtypes, which have revealed a remarkable degree of heterogeneity within a single IN class [28*,40].

If MN identity plays an instructive role in shaping the specificity of premotor inputs, then one might expect to observe differences in the types of premotor INs that target a specific MN type. The development of rabies-based transynaptic tracing methods has enabled the mapping of inputs onto MNs with high resolution [41,42]. Analyses of the patterns of premotor connectivity have revealed striking differences in the types and distribution of INs that MN subtypes engage.

LMC neurons receive inputs that are biased towards *Lbx1*⁺ spinal inhibitory INs localized ipsilateral to the target muscle (Figure 2B) [43**]. In contrast, MNs projecting to axial muscles, including those located in the MMC and HMC, receive inputs that are distributed across both sides the spinal cord, with a bias towards contralateral populations.

Differences in the distribution premotor INs are also observed for inputs onto MN pools that occupy specific regions within the LMC. LMC neurons situated ventromedially receive a greater proportion of inputs from contralateral premotor INs than MNs projecting to distal limb muscles, which are more dorsolaterally positioned [43**]. How these differences in the distribution of premotor inputs are determined is not currently known. A possible mechanism is through the configuration of MN dendrites. MMC neurons, which are positioned medially, have dendrites which extend across the midline and can capture a greater proportion of inputs originating from contralateral IN populations (Figure 2B). Recent studies also show a bias in the types of INs that synapse onto MNs projecting to extensor and flexor muscles [39*]. Given that MN intrinsic factors such as *Pea3* are known influence MN dendritic morphology [33], inputs from premotor INs could be shaped by both MN position and dendritic architectures.

Motor neurons as determinants of connectivity and function in locomotor networks

If MNs function only as passive recipients of rhythmic signals provided by premotor networks, the output of CPGs would be predicted to be unaffected by mutations that disrupt MN columnar and pool identities. Two recent studies have explored the role of MN columnar identity in CPG output, using genetically encoded calcium indicators to measure fictive locomotion in *Foxp1* mutants [44**,45**]. Removal of *Foxp1* selectively from MNs leads to a transformation of LMC neurons to an axial-MN identity, but these MNs still target limb muscles. In the absence of *Foxp1*, core outputs of CPG circuits are retained, including the normal rhythmic firing pattern of MNs and strict left-right alternating pattern of bursts.

Locomotor CPGs therefore can provide rhythmic activation and L-R alternation to MNs, irrespective of MN subtype identity.

In tetrapods, spinal CPGs are also essential for the reciprocal activation of MNs targeting limb flexor and extensor muscles. In *Foxp1* mutants, extensor firing patterns are lost and MNs display a predominantly flexor-like activity (Figure 2C) [44**,45**]. Mutation of *Foxp1* also shifts MNs to a more ventromedial position, raising the possibility that the defects are a consequence of altered MN position. Hinckley et al. further tested this model by misexpressing the Lim homeodomain factor *Lhx3* in all MNs, a manipulation that suppresses LMC differentiation and promotes an MMC identity [15,46]. Some ectopic MMC neurons occupy the same position as LMC neurons, but still display a predominantly flexor-like MN bursting pattern [44**]. These observations suggest that position alone is not sufficient to determine the E-F pattern of MN firing, and that MN-derived cues determine input specificity from premotor CPGs.

Analysis of CPG activities in *Foxp1* mutants also provides insight into how limb-based locomotor circuits evolved. The perseverance of MN bursting and L-R alternation in *Foxp1* mutants is consistent with the idea that spinal CPG circuits originated in primitive vertebrates lacking limbs [47,48]. Studies in rodents show that the bursting of rostral lumbar flexor LMC neurons represents a caudal extension of a wave of firing originating from thoracic segments [49]. This observation is in agreement with a model in which bursting of limb flexor MNs evolved from cooption of CPGs used to activate axial muscles during undulatory locomotor behaviors. How the extensor CPG circuit evolved is unclear, but studies in zebrafish suggest the E-F CPG may have been co-opted from premotor circuits used for postural correction [50*].

In addition to contributing to the pattern of output from E-F CPGs, MNs have been recently demonstrated to influence the firing properties of excitatory premotor INs. In zebrafish, MNs involved in slow, intermediate, a fast swimming were shown to be electrically coupled to V2a INs through gap junctions [51**]. V2a INs provide a major excitatory input to MNs and are a key component of the rhythm-generating networks that drive undulatory locomotion [26,52]. Hyperpolarization of MNs increases the firing threshold and decreases the firing frequency of V2a INs. Conversely, depolarization of MNs decreases V2a thresholds and increases their firing frequency. Backward propagation of electrical signals from MNs therefore can have an immediate influence on the activities of motor networks, by setting the threshold and firing properties of V2a INs.

Conclusions

Studies investigating the contribution of MNs to locomotor circuit assembly have uncovered critical roles in sensory-motor synaptic specificity and coordinating patterns of motor output. If MN identity contributes to premotor IN connectivity, how might this work at a cellular level? MN subtypes could express recognition molecules that directly influence the connectivity with premotor INs (Figure 2D). The same surface molecules known to govern MN organization, such as type II cadherins, could play additional roles in shaping central synaptic specificity. In the visual system cadherins have been demonstrated to be critical for

target specificity [53], but cadherin function in spinal circuit assembly remains to be determined. LMC neurons are known to produce secreted morphogens including retinoic acid [54], that may act on premotor INs to drive circuit connectivity (Figure 2D). In the future, it will be informative to reevaluate genetic manipulations known to affect MN differentiation and resolve how MN identity influences central connectivity within spinal circuits.

While MN subtype identity appears to play an important role in determining aspects of spinal circuit connectivity and function, studies on IN subtypes have also revealed insights into how dynamic locomotor patterns as well as fine motor skills are controlled. Recent studies have highlighted the modular nature of premotor circuits, indicating diverse IN populations can contribute to seemingly simple patterns of output, such as L-R alternation [34,55*]. Mapping of the premotor neurons originating from supraspinal neurons in the brainstem has also uncovered fundamental insights into how fine limb control is achieved [56,57]. Whether connections from higher descending pathways to spinal motor networks are instructed by MN subtype identity remains to be determined.

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Highlights

Evolution of locomotor behaviors correlates with changes in motor neuron organization

Motor neuron position can facilitate spinal circuit synaptic specificity

Subtype-specific cues provided by motor neurons may instruct circuit assembly

Motor neurons can influence premotor network activity

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Figure 1. Organization and subtype diversification of spinal MNs

(A) Spinal motor neurons targeting groups of related muscles are organized in columns. Two motor columns are shown. Medial motor column (MMC) neurons express the Lim homeodomain protein Lhx3 and project to dorsal epaxial muscles. Lateral motor column (LMC) neurons express Foxp1, target limb muscle, and are found at brachial and lumbar spinal levels.

(B) Comparison of motor neuron organization in *Foxp1* and *Pbx* mutants. At brachial levels MNs are organized in either the LMC or MMC. LMC neurons further differentiate into motor pools that target single limb muscles. In *Foxp1* mutants the LMC is transformed to a hypaxial motor column (HMC) identity. These HMC neurons target limb muscles but are dispersed within the motor column, indicating a loss of motor pool identity. In *Pbx* mutants HMC and MMC neurons are unclustered and disordered.

(C) Genetic manipulations that affect MN columnar organization. For simplicity only LMC, HMC, MMC, and preganglionic column (PGC) neurons are shown. Top panels show columnar organization along the rostrocaudal axis, bottom panels intrasegmental columnar position at brachial and thoracic levels. In *Foxp1* mutants LMC and PGC neurons are transformed to an HMC fate, but the remaining columns are organized. In combined *HoxA/HoxC* cluster mutants, brachial LMC and PGC neurons are lost. In *Hoxc9* mutants, thoracic HMC and PGC neurons are transformed to an LMC fate due to derepression of brachial *Hox*

genes. In *Pbx* mutants all Hox-dependent programs of MN differentiation are lost, and the remaining subtypes are interspersed.

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Figure 2. Motor neurons as determinants of synaptic specificity in locomotor circuits

(A) Motor neuron identity and position influences the specificity of connections between proprioceptive sensory neurons and motor neurons. In wildtype (*wt*) animals sensory and motor neurons targeting the same muscle peripherally establish central monosynaptic connections. In *Foxp1* mutants, proprioceptive sensory neurons target the same dorsoventral position and synapse with MNs, irrespective of the muscle target of the MN. In *Sema3e* mutants, the absence of inhibitory signals from MN causes sensory neurons to target the inappropriate MN pool.

(B) Pattern of premotor interneuron inputs to specific MN subtypes. LMC neurons receive a majority of their presynaptic inputs from interneurons located ipsilaterally. MMC neurons receive inputs from interneurons located on both sides of the spinal cord. The greater proportion of contralateral inputs to MMC neurons may be due to the extension of MMC dendrites across the midline of the spinal cord.

(C) Motor neuron subtype identity influences the pattern of output from locomotor CPG networks. Loss of LMC identity in conditional *Foxp1* mutants and *Lhx3-ON* mice leads to a loss of reciprocal extensor-flexor output from locomotor CPGs, with the firing of the remaining MNs displaying a flexor-like pattern. In *Foxp1* mutants MNs are shifted to a more ventromedial position, whereas in *Lhx3-ON* mice, MNs still occupy an LMC-like position. Both manipulations lead to loss of extensor firing pattern, suggesting MN position is not critical for establishing this pattern of output. *Foxp1* is also expressed in a subset of V1 INs, and whether this expression is affected after *Olig2::Cre*-mediated deletion is unclear.

(D) Models for the influence of MN subtype identity on premotor circuit connectivity. LMC neurons could provide a source of instructive signals that direct connectivity with ipsilateral premotor interneuron populations. These signals could affect the differentiation of premotor interneurons or influence interneuron connectivity with MNs. MN subtypes may also express recognition molecules (RecA, RecB) that dictate premotor synaptic specificity.