Symposium

Molecular and Cellular Mechanisms of Motor Circuit Development

Paschalis Kratsios,[1,2] Niccolò Zampieri,[3] Robert Carrillo,[2,4] Kota Mizumoto,[5] Lora B. Sweeney,[6] and Polyxeni Philippidou[7]

[1]Department of Neurobiology, University of Chicago, Chicago, Illinois 60637, [2]Neuroscience Institute, University of Chicago, Chicago, Illinois 60637, [3]Max-Delbrück-Center for Molecular Medicine in the Helmholtz Association (MDC), Berlin 13125, Germany, [4]Department of Molecular Genetics and Cell Biology, University of Chicago, Chicago, Illinois 60637, [5]Department of Zoology, University of British Columbia, Vancouver, British Columbia V6T 1Z3, Canada, [6]Institute of Science and Technology Austria, Klosterneuburg 3400, Austria, and [7]Department of Neurosciences, Case Western Reserve University, Cleveland, Ohio 44106

Motor circuits represent the main output of the central nervous system and produce dynamic behaviors ranging from relatively simple rhythmic activities like swimming in fish and breathing in mammals to highly sophisticated dexterous movements in humans. Despite decades of research, the development and function of motor circuits remain poorly understood. Breakthroughs in the field recently provided new tools and tractable model systems that set the stage to discover the molecular mechanisms and circuit logic underlying motor control. Here, we describe recent advances from both vertebrate (mouse, frog) and invertebrate (nematode, fruit fly) systems on cellular and molecular mechanisms that enable motor circuits to develop and function and highlight conserved and divergent mechanisms necessary for motor circuit development.

Key words: **development; interneurons; motor circuits; motor neurons; spinal cord; synaptic specificity; terminal selectors**

Introduction

Motor neurons (MNs) represent the main output of the central nervous system. In humans, MNs constitute the cellular substrates for several movement disorders. Due to their stereotypic cell body position, easily identifiable axons, and highly precise synaptic connections with well-defined muscles, MNs are exceptionally well characterized in many invertebrate and vertebrate model systems.

The combination of multiple model organisms has been a powerful and fruitful approach in the study of motor circuits. Classical anatomical and functional studies in chick embryos (Landmesser, 2018), lamprey (Grillner, 2006), *Xenopus* (Borodinsky, 2017), and cats (Jankowska and Hammar, 2002) have profoundly shaped our current understanding of motor circuit development and function. Genetically tractable model organisms, such as worms and flies, have provided valuable insights into the molecular mechanisms that control MN specification, connectivity, and maintenance (Thor and Thomas, 2002; Catela and Kratsios, 2021), while studies in mice and zebrafish have helped delineate the diversity and function of the major classes of neurons within the spinal cord (Goulding, 2009; Fetcho and McLean, 2010; Grillner and El Manira, 2020).

Recent technological advances, such as single-cell RNA sequencing and viral tracing tools, have enabled the examination of motor circuit development and connectivity with unprecedented spatial and temporal resolution. In this SfN minisymposium review, we combine insights from both invertebrate and vertebrate motor systems to uncover principles that govern motor circuit development, assembly, evolution, and maintenance.

Early Steps in Vertebrate MN Specification and Connectivity

In mammals, MNs can be subdivided into several dozens of molecularly, anatomically, and functionally distinct subtypes. The precise specification of MN subtype identity during development is a critical first step in the assembly of motor circuits. Morphogen gradients acting along the dorsoventral axis of the neural tube give rise to distinct progenitor domains through the induction of homeodomain transcription factors (Jessell, 2000). MNs are derived from a ventral progenitor domain expressing Olig2, whereas other ventral and dorsal progenitor domains give rise to interneuron populations with diverse functions in motor control (Sengupta and Bagnall, 2023; Fig. 1*A*). Along the rostrocaudal axis, MN diversification is further mediated by opposing gradients of retinoic acid and fibroblast growth factor that establish the expression domains of Hox transcription factors (Philippidou and Dasen, 2013). For example, at cervical levels of the spinal cord, Hox5 proteins mediate the development of phrenic MNs that innervate the diaphragm (Philippidou et al., 2012; Vagnozzi et al., 2020), while a network

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Correspondence should be addressed to Paschalis Kratsios at pkratsios@uchicago.edu or Polyxeni Philippidou at pxp282@case.edu.

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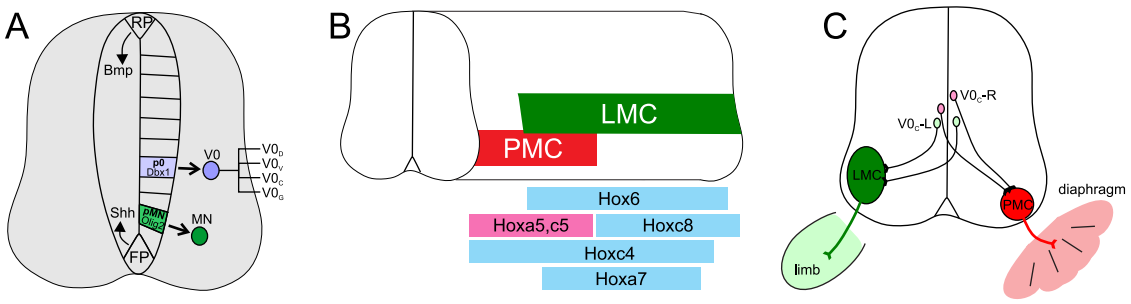

Figure 1. Neuronal diversity in the mammalian spinal cord. ***A***, Morphogen gradients acting along the dorsoventral axis of the neural tube generate progenitor domains that give rise to the major neuronal classes in the spinal cord, which are further subdivided into more diverse subtypes. RP, roof plate; FP, floor plate; Shh, Sonic hedgehog; Bmp, bone morphogenetic proteins. ***B***, Along the rostrocaudal axis of the spinal cord, Hox proteins further diversify MNs. For example, at rostral levels of the spinal cord (cervical–brachial), Hox5 proteins control the development of phrenic (PMC) neurons while a network of ∼20 Hox proteins establishes limb-innervating (LMC) MN identities. ***C***, Distinct subsets of cholinergic V0 neurons target either LMC or PMC neurons. The mechanisms that distinguish locomotor ($V0_C$–L) from respiratory ($V0_C$–R) $V0_C$ neurons and dictate their selective targeting are currently unknown.

of ∼20 Hox proteins establishes limb-innervating MN identities (Dasen et al., 2005; Fig. 1*B*). The establishment of unique transcription factor codes for each MN subtype endows MNs with distinct features, such as axonal trajectory and cell body position.

The acquisition of cell-type identity is closely coupled to the selection of axonal trajectories and the targeting of MNs to their correct targets in the periphery. Transcriptional programs within specific MN subtypes direct the expression profiles of guidance receptors that determine MN responsiveness to attractive and repulsive cues (Catela et al., 2015). For example, LIM homeodomain transcription factors drive the expression of ephrin receptors that sensitize limb-innervating motor axons to repulsive ephrin ligands in the mesenchyme and ultimately determine their trajectory into either the dorsal or ventral limb (Helmbacher et al., 2000; Kania et al., 2000; Kania and Jessell, 2003; Luria et al., 2008). These receptor–ligand interactions, in combination with activity-dependent mechanisms (Hanson and Landmesser, 2004; Landmesser, 2018) and signals from adjacent tissues (Martins et al., 2022; Vieira et al., 2022), sculpt MN peripheral connectivity. While we understand MN axon guidance at several key points, such as the binary selection point between the dorsal and ventral limb mesenchyme, the mechanisms that control the targeting of distinct MN subsets to single muscles are still not well defined. We also know significantly less about how distinct interneuron classes target specific MN populations to form the local microcircuits that oversee different aspects of motor control, such as flexor and extensor muscle alternation (Tripodi et al., 2011; Ronzano et al., 2022).

Conserved Mechanisms of MN Maintenance

Extensive research over the past decades has focused on the early steps of MN development, making seminal contributions to our understanding of the molecular mechanisms (e.g., morphogens, LIM proteins, HOX proteins) that control specification of progenitor cells, generation of young postmitotic MNs, and circuit assembly. Unlike early development, studies of the molecular mechanisms that control the final steps of MN differentiation, during which postmitotic MNs acquire their function-defining features, such as neurotransmitter synthesis, electrical activity, and signaling properties, are still in the early stages. Furthermore, and perhaps most important, the mechanisms that ensure maintenance of such features throughout postnatal life remain largely unknown.

During the last steps of their differentiation, postmitotic MNs are instructed to express batteries of terminal identity genes (often called "effector genes") that code for proteins that determine MN functional properties throughout life. These proteins include enzymes and transporters for neurotransmitter biosynthesis, neurotransmitter receptors, ion channels, neuropeptides, and other signaling molecules. Terminal identity genes are continuously expressed from the last steps of development throughout adulthood, forming a distinct molecular signature for postmitotic MNs that uniquely defines their terminal identity and function.

Transcriptional mechanisms that establish and maintain MN terminal identity

Terminal selectors are transcription factors that are expressed continuously from development through adulthood in specific neuron types (Hobert, 2008, 2016a,b). They can act in combinations to establish and maintain neuronal terminal identity throughout life. Mechanistically, terminal selectors bind directly to the *cis*-regulatory regions of neuron type-specific terminal–identity genes to activate their transcription. Terminal selectors are highly conserved. They have been identified in the nervous system of nematodes (*Caenorhabditis elegans* [*C. elegans*]), fruit flies (*Drosophila*), cnidarians (*Nematostella vectensis*), marine chordates (*Ciona intestinalis*), and mice (*Mus musculus*; Hobert and Kratsios, 2019). Although terminal selectors have been described for various neuron types across species, terminal selectors for MNs have only been described in *C. elegans* and mouse motor circuits.

Terminal selectors of MN identity in *C. elegans*

The *C. elegans* ventral nerve cord (analogous to the vertebrate spinal cord) contains five classes of cholinergic (DA, DB, VA, VB, AS) and two classes of GABAergic (DD, VD) MNs, which are essential for locomotion (Von Stetina et al., 2006; Fig. 2*A*).

UNC-3, the sole *C. elegans* ortholog of the COE (Collier/Olf/Ebf) family of transcription factors (Prasad et al., 1998; Dubois and Vincent, 2001), acts as a terminal selector in nerve cord MNs, where it directly activates the expression of dozens of terminal identity genes (e.g., neurotransmitter biosynthesis components, neurotransmitter receptors, ion channels, and neuropeptides; Kratsios et al., 2011; Li et al., 2020; Li and Kratsios, 2021; Fig. 2*B*). Using an auxin-inducible degradation method for UNC-3 depletion selectively in adult cholinergic MNs, two recent studies reported adult-specific loss of expression of various UNC-3 target genes (Kerk et al., 2017; Li et al., 2020). Altogether, the terminal selector UNC-3 (Collier/Ebf) initiates during

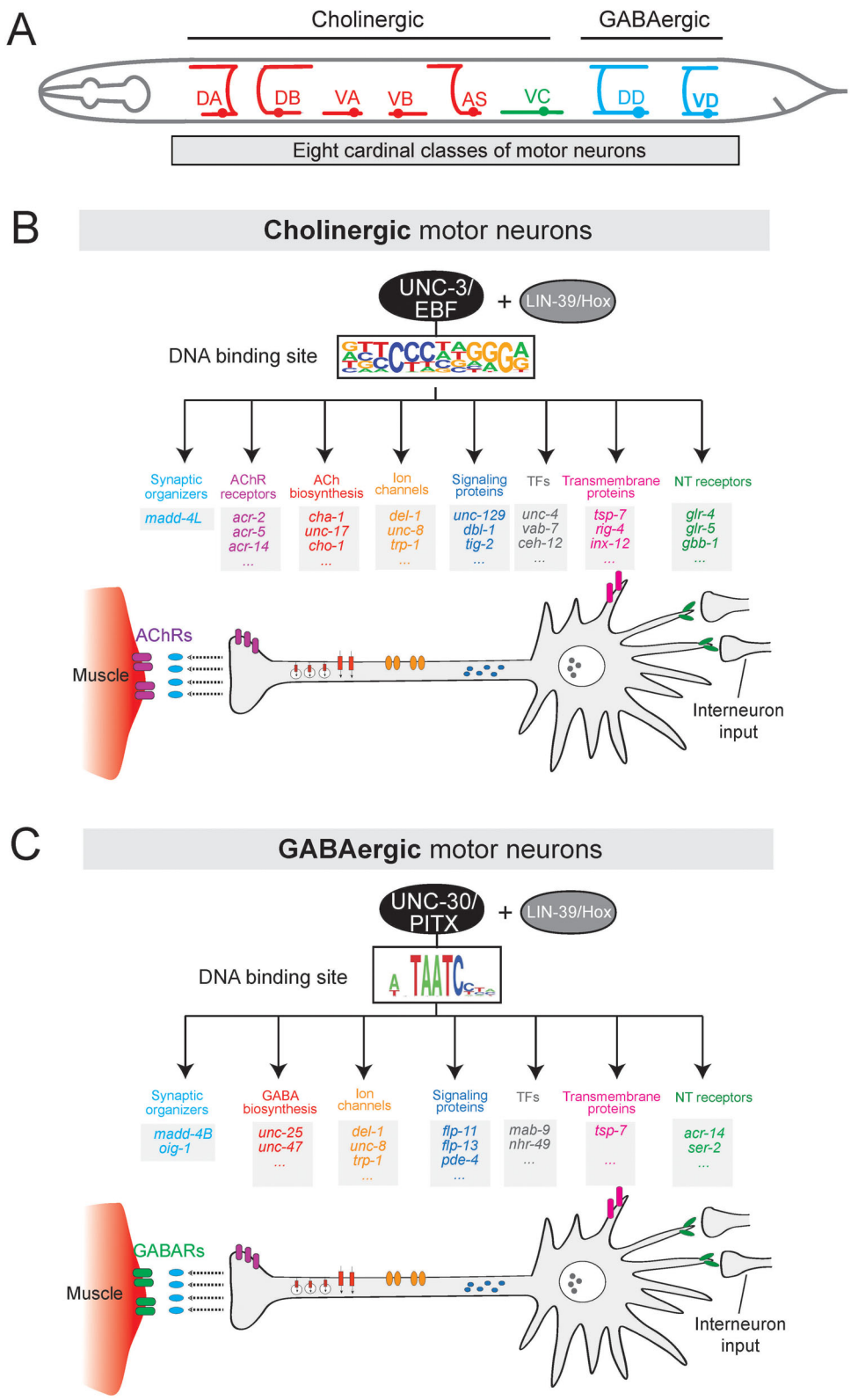

Figure 2. Terminal selectors of MN identity in *C. elegans*. ***A***, Eight cardinal classes of MNs are in the *C. elegans* ventral nerve cord, six are cholinergic, and two are GABAergic. Seven classes (DA, DB, VA, VB, AS, DD, VD) control locomotion, whereas VC neurons control egg laying. ***B***, UNC-3/EBF functions as a terminal selector of cholinergic MN identity in five cholinergic classes (DA, DB, VA, VB, AS). UNC-3/EBF collaborates with LIN-39/HOX to coactivate scores of MN terminal identity genes. The DNA-binding site of UNC-3 is adapted from Li et al. (2020). ***C***, UNC-30/PITX functions as a terminal selector of GABAergic MN identity in two GABAergic classes (DD, VD). UNC-30/PITX collaborates with LIN-39/HOX to coactivate scores of MN terminal identity genes (Feng et al., 2020). The DNA-binding site of UNC-30 is adapted from Cinar et al. (2005).

development and maintains throughout life the expression of numerous terminal identity genes in cholinergic MNs (Fig. 2*B*). The locomotor and MN defects of *unc-3* mutant nematodes can be rescued by providing mouse *Ebf1* or *Ebf2* in MNs, indicating high functional conservation between *C. elegans* UNC-3 and mouse *Ebf1/2* (Catela et al., 2019). Supporting the evolutionary conservation of its terminal selector function, a chordate ortholog of UNC-3, *Ciona robusta* COE, is both required and sufficient for inducing cholinergic MN fate (Kratsios et al., 2011). In the vertebrate nervous system, Ebf transcription factors control neurogenesis, as well as neuronal migration and differentiation (Garcia-Dominguez et al., 2003; S. S. Wang et al., 2004; Green and Vetter, 2011; Chiara et al., 2012; Monahan et al., 2017), but their function in MNs remains unknown. Future work is needed to address this gap, as mutations in human *EBF3* cause a neurodevelopmental syndrome characterized by motor developmental delay (Chao et al., 2017; Sleven et al., 2017).

UNC-30 is the sole *C. elegans* ortholog of the PITX family of transcription factors, mutations in which affect eye development in humans (Tran and Kioussi, 2021). UNC-30 is expressed in the two classes of GABAergic (DD, VD) MNs, in which it acts as terminal selector by activating a host of terminal identity genes (Jin et al., 1994; Eastman et al., 1999; Cinar et al., 2005). Through inducible protein depletion experiments, it was recently shown that UNC-30/PITX is continuously required to activate expression of GABA biosynthesis genes (Correa et al., 2024; Fig. 2*C*).

The highly conserved family of Hox transcription factors has been primarily associated with early development roles, such as anterior–posterior patterning of the embryo and the nervous system (Mallo et al., 2010; Lacombe et al., 2013). However, recent studies in the *C. elegans* nervous system have uncovered noncanonical roles for Hox genes during later stages of nervous system development and adult life, broadening the functional repertoire of this fundamental family of transcription factors beyond early patterning. Accumulating evidence strongly suggests that *lin-39* (*Scr/Dfd/Hox4-5*) acts as a terminal selector of cholinergic MN identity in the midbody region of the *C. elegans* ventral nerve cord (Feng et al., 2020, 2022). ChIP-sequencing revealed that LIN-39, like UNC-3, binds directly at its terminal identity gene targets. Furthermore, LIN-39 is continuously required to maintain the terminal identity of nerve cord MNs (Feng et al., 2020, 2022). Hence, LIN-39 acts as a bona fide terminal selector that works with UNC-3 to establish and maintain cholinergic MN identity in the midbody region (Fig. 2*B*). Interestingly, LIN-39 also collaborates with UNC-30, the terminal selector of GABAergic MNs (Fig. 2*C*; Feng et al., 2020, 2022). We note that in MNs located posterior to the nerve cord, the posterior Hox gene *egl-5/Abd-B/Hox9*-13 also acts with UNC-3 to control MN terminal identity (Kratsios et al., 2017). Hence, the terminal identity of *C. elegans* MNs is achieved via an intersectional strategy, as UNC-3 collaborates with region-specific Hox proteins.

Terminal selectors coordinate MN identity and synaptic connectivity

In addition to MN terminal identity defects, recent studies showed that *unc-3* and *unc-30* mutant animals display MN connectivity defects (Howell et al., 2015; Kratsios et al., 2015; Barbagallo et al., 2017; Correa et al., 2024). The neuromuscular synapse defects observed in *unc-3* mutants can be partially explained by UNC-3/Ebf directly regulating the expression of the long isoform of *madd-4* (Punctin/*ADAMTSL*), a secreted synapse-organizing molecule required for ACh receptor clustering on the muscle. Intriguingly, UNC-30/PITX acts in an analogous manner in GABAergic MNs by regulating the expression of the short *madd-4* isoform that is necessary for GABA receptor clustering (Correa et al., 2024). These studies on *C. elegans* MNs expand the definition of terminal selectors: they not only regulate effector genes critical for neurotransmitter biosynthesis and neuronal signaling (e.g., ion channels) but also control synaptic connectivity via the regulation of distinct synapse organizers.

Terminal selectors of MN identity in mice

In mice, RNA sequencing of isolated spinal MNs at early postnatal stages revealed sustained expression of multiple Hox genes in brachial (*Hoxc4*, *Hoxa5*, *Hoxc5*, *Hoxa6*, *Hoxc6*, *Hoxa7*, *Hoxc8*), thoracic (*Hoxd9*), and lumbar (*Hoxa10*, *Hoxc10*, *Hoxa11*) MNs (Catela et al., 2022). Genetic removal of *Hoxc8* at early and late developmental stages resulted in significant downregulation of various terminal identity genes (*Nrg1*, *Mcam*, *Pappa*) and motor defects (Catela et al., 2022), indicating that *Hoxc8* is continuously required for MN terminal identity and function. Hence, Hoxc8 is a candidate terminal selector for spinal MNs of the brachial domain. Similarly, *Hox5* genes are continuously required in mouse spinal MNs responsible for breathing (Philippidou et al., 2012), raising the possibility that distinct Hox proteins, in addition to their early roles in MN development, may act at later stages as terminal selectors in different subtypes of spinal MNs.

Another terminal selector candidate is the LIM homeodomain protein *Islet1* (*Isl1*), which is required for early induction of genes necessary for ACh biosynthesis in mouse spinal MNs and the in vitro generation of MNs from human pluripotent stem cells (Cho et al., 2014; Qu et al., 2014; Rhee et al., 2016). Interestingly, *Isl1* is expressed continuously in brachial MNs and amplifies its own expression (Erb et al., 2017)—both defining features of a terminal selector gene. However, inducible gene inactivation experiments are needed in the adult to determine whether Isl1 is required to maintain the terminal identity program of spinal MNs.

Functional Organization of Motor Circuits in Vertebrates

The ability to generate coordinated and adaptable movements is a defining feature of the nervous system. While planning and initiation of motor behaviors takes place in the brain, the spinal cord orchestrates the execution of a wide variety of motor programs and reflexive actions (Arber and Costa, 2018). In vertebrates, spinal circuits integrate motor commands and sensory information to activate functionally coherent ensembles of MNs in order to produce precise sequences of muscle contraction to control movement and posture (Kiehn, 2016).

The generation of movement in limbed vertebrates relies on three fundamental levels of regulation. First, alternation and cocontraction of flexor and extensor muscles control joint movement. Second, the timing of left and right limb activation underlies the generation of motor patterns. Finally, intersegmental coordination of forelimb, trunk, and hindlimb muscles is necessary for generating different gaits and complex motor sequences, as well as maintaining balance and postural control. Spinal interneurons oversee this remarkable functional complexity by assembling into circuits that control patterns and rhythms of muscle activation across the spinal cord, thus providing the fundamental neural substrate for a vast repertoire of movements ranging from relatively simple locomotor activities like walking and swimming to more complex motor skills like grasping and

object manipulation (Osseward and Pfaff, 2019). Thus, defining the functional organization of spinal neurons is a key step toward understanding the circuit logic governing motor control.

Since the discovery of the transcriptional regulatory network controlling the development of the mammalian spinal cord, neurons have been classified according to the cardinal progenitor zones of origin that are set up along the dorsoventral and rostrocaudal axes through the action of opposing gradients of morphogens (Sagner and Briscoe, 2019). These findings have paved the way for the genetic dissection of neuronal function in the mouse spinal cord, providing important insights into the roles of different classes in key aspects of motor control, such as pattern and rhythm generation (Bikoff, 2019). The revolution in single-cell and transcriptome analysis technologies has highlighted that cardinal classes are composed of heterogeneous populations of neurons presenting distinct molecular, anatomical, and physiological features, indicating that a higher level of complexity underlies the functional organization of spinal motor circuits (Russ et al., 2021). A notable example is provided by recent data showing that mouse cardinal interneuron classes can be further fractioned into subclasses defined by genetic signatures indicating their connectivity range, either local or long distance (Osseward et al., 2021). Temporal mechanisms related to timing of neurogenesis and transcription factor expression are at the basis of this distinction, with early born neurons projecting long distance and late born locally. While markers identifying cardinal class or connectivity in isolation are not sufficient to precisely define the postsynaptic target of a neuron, the intersection of the two can be predictive of circuit organization, thus indicating that during development, orthogonal genetic programs are superimposed to impart spinal neurons with defining aspects of their functional identity.

While the organization of local circuits controlling flexor/extensor and right/left alternation has been extensively studied, comparatively less is known about the circuits coordinating the activity of different levels of the spinal cord. Interneurons connecting distant segments were first described by Sherrington and Laslett (1903). These neurons were termed propriospinal neurons and have been proposed to play a crucial role in motor control and sensory integration (Laliberte et al., 2019). Anatomical and physiological studies have demonstrated the existence of both ascending and descending subtypes and confirmed their important roles in the integration and propagation of sensorimotor information (Ruder et al., 2016; Pocratsky et al., 2020). However, a systematic functional characterization of propriospinal circuits is not available yet. In order to start filling this gap, the Zampieri lab is studying the functional organization of neurons interconnecting cervical, thoracic, and lumbar segments of the mouse spinal cord. In unpublished work, we identified anatomically and functionally distinct subtypes of propriospinal neurons belonging to cardinal classes of spinal interneurons, thus supporting the idea that the intersection of classical cardinal identities and connectivity range is a defining factor in controlling spinal neuron diversification.

Distinct Spinal Interneuron Functions in Locomotor and Respiratory Control in Mammals

Limb-innervating MNs receive extensive local interneuron inputs that control aspects of movement such as left/right and flexor/extensor alternation. While the organization, connectivity, and function of spinal interneuron populations in the modulation of locomotor circuits have been extensively studied (Dougherty, 2023; Sengupta and Bagnall, 2023), considerably less is known about propriospinal respiratory circuits. Recent rabies virus tracing experiments suggested that few direct inputs onto diaphragm-innervating phrenic MNs originate in the spinal cord (Wu et al., 2017), although tracing experiments with polysynaptic viruses reveal much more intricate spinal respiratory circuits (Lane et al., 2008). In addition, excitatory interneurons at cervical and thoracic levels of the spinal cord are able to sustain breathing after spinal cord injury (Cregg et al., 2017; Satkunendrarajah et al., 2018; Jensen et al., 2024), suggesting important functions for spinal interneurons in breathing regulation (Zholudeva et al., 2018; Jensen et al., 2019; Streeter et al., 2020; Sunshine et al., 2020); however, the function of genetically defined interneuron classes in distinct aspects of respiratory control is not well understood. Ongoing work from the Philippidou lab seeks to define the function, connectivity, and molecular profile of spinal respiratory interneuron populations.

A class of cholinergic interneurons, $V0_C$ interneurons, which are derived from the Dbx1-expressing p0 domain and express the transcription factor Pitx2, give rise to large cholinergic synapses (C-boutons) on MN cell bodies (Zagoraiou et al., 2009). These interneurons increase MN excitability to ensure that a sufficient motor output is generated during demanding motor tasks, such as swimming (Miles et al., 2007; Zagoraiou et al., 2009). While cholinergic Pitx2+ interneurons consist of a relatively small population, there are several indications that there might be considerable diversity among them. Within locomotor circuits, there are at least two populations, one of which projects exclusively to ipsilateral targets and the other of which projects bilaterally, indicating that distinct programs underlie their connectivity (Zagoraiou et al., 2009; Stepien et al., 2010). Cholinergic synapses on different MN subtypes also exhibit differential clustering of certain ion channels (Deardorff et al., 2013). Single-nucleus RNA sequencing from the adult spinal cord revealed eight classes of cholinergic interneurons, two of which expressed Pitx2 (Alkaslasi et al., 2021). In unpublished work, the Philippidou lab found that distinct Pitx2+ respiratory interneurons at cervical levels of the spinal cord directly project to phrenic MNs and regulate the adaptive control of breathing (Fig. 1C).

The segregation of closely related interneurons into distinct functional circuits suggests that they must target specific MN subtypes during development in order to become integrated into either respiratory or locomotor circuits. Although specific connectivity within motor circuits is essential for the execution of motor actions, deciphering the underlying logic of motor circuit assembly remains a considerable challenge.

Mechanisms of Motor Circuit Wiring and Synaptic Specificity in Vertebrates

Motor circuits must assemble with high fidelity during development. This is especially critical for respiratory circuits that need to initiate and sustain robust breathing immediately upon birth. Unlike well-organized, laminar sensory structures, such as the retina, that provide an orderly blueprint to facilitate connectivity, the complex architecture of brainstem and spinal cord motor circuits presents a particular challenge for the exquisite partner selection required to execute motor behaviors. To date, we know surprisingly little about the molecular mechanisms that underlie synaptic specificity within motor circuits.

In vertebrate sensory-motor circuits, the correct positioning of MNs and spatial features of the MN dendritic tree, such as the angle of interaction with approaching axons, appear to be

critical for their correct targeting by sensory axons (Vrieseling and Arber, 2006; Surmeli et al., 2011; Balaskas et al., 2019). However, gene mutations that alter molecular identity but not cell body position dramatically reconfigure MN inputs, indicating that MN position is unlikely to be the only critical parameter for MN connectivity (Pecho-Vrieseling et al., 2009; Fukuhara et al., 2013; Hinckley et al., 2015; Machado et al., 2015). Rather, topography-based strategies may act as a critical first step to limit access to the available number of MN synaptic partners (Balaskas et al., 2020). In addition, MN subtype-specific molecular identity, established by early transcriptional programs, has emerged as a critical determinant of sensory-motor connectivity (Baek et al., 2017; Dasen, 2017; Shin et al., 2020; Imai et al., 2021).

To date, the construction of the monosynaptic stretch reflex, where Group Ia sensory afferents synapse directly onto the alpha MNs innervating the same muscle, remains the best-studied example of how molecular recognition systems, with cell-specific surface receptor and ligand pairs driving largely repulsive interactions, contribute to synaptic specificity in the mammalian spinal cord (Pecho-Vrieseling et al., 2009). A notable exception is the well-orchestrated assembly of a GABAergic presynaptic inhibitory circuit that filters sensory information transfer to MNs. Within this circuit, a subset of GABAergic interneurons form synapses with the terminals of proprioceptive sensory neurons with stringent selectivity through both cell–surface adhesive interactions and activity-dependent mechanisms (Betley et al., 2009; Ashrafi et al., 2014; Mende et al., 2016). Despite these well-defined examples, elucidating mechanisms of synaptic specificity in locomotor circuits that rely on complex local circuits and the coregulation of multiple muscles remains daunting.

Motor circuits that mediate breathing, on the other hand, are comparatively simple and have a robust, reproducible and easily measurable outcome, making them an ideal model for understanding the relationship between genetic networks, neural connectivity, and function. Mammals have evolved a sophisticated motor program to support continuous, yet adaptable, breathing. Despite the complex circuitry that ensures breathing adaptability and modulation, at the core of respiratory networks is a simple tripartite motor circuit that drives diaphragm muscle contractions during inspiration. Brainstem neurons within the pre-Bötzinger complex (preBötC) generate the rhythmic pattern of breathing and project to rostral ventral respiratory group (rVRG) neurons that activate phrenic MNs in the spinal cord to initiate diaphragm contractions. Connections among respiratory neurons must form without errors: failure to do so leads to breathing failure and death.

Despite the critical importance of respiratory motor circuits for survival, the mechanisms that underlie their connectivity are not well defined. The Philippidou lab identified a set of cadherins that are essential for establishing the connectivity between rVRG neurons and phrenic MNs. Cadherins can be classified as either Type 1 or Type 2 based on the sequence and structure of their extracellular domain, and each class was thought to function independently. Surprisingly, however, we discovered that coordinated activity of a Type 1 cadherin (*N*-cadherin) and Type 2 cadherins (Cadherin-6, 9, and 10) is required in MNs to generate robust respiratory motor output. MN-specific cadherin inactivation in mice results in perinatal lethality due to respiratory failure and a striking reduction in phrenic MN bursting activity. This combinatorial cadherin code is required to establish phrenic MN cell body and dendritic topography; surprisingly, however, cell body position appears to be dispensable for the targeting of phrenic MNs by descending respiratory inputs. These findings demonstrate that Type 1 and Type 2 cadherins function cooperatively to generate a robust breathing output and reveal novel, topography-independent, strategies that drive the assembly of motor circuits (Vagnozzi et al., 2022). Cadherin signaling is only required during a narrow developmental window to establish proper respiratory circuit output (Vagnozzi et al., 2023), revealing that temporally distinct mechanisms underlie respiratory circuit development and maintenance.

Mechanisms of Motor Circuit Wiring and Synaptic Specificity in Invertebrates

While work on mouse locomotor and respiratory circuits has provided valuable insights into mechanisms of circuit assembly, fundamental questions still remain about the relative contributions of anatomical organization and molecular programs to synaptic specificity and the pathways that link early transcriptional programs to neuronal connectivity, largely due to the complexity of mammalian motor circuits. "Simpler" nervous systems have provided fundamental insights into the processes that instruct neural circuit assembly. While the *Drosophila* nervous system has significantly fewer neurons than the human brain, each fly neuron must similarly be instructed to recognize its appropriate postsynaptic target(s). This process is akin to the chemoaffinity hypothesis proposed by Roger Sperry over 60 years ago (Sperry, 1963). Various cell surface proteins, including cadherins, leucine-rich repeat proteins, and immunoglobulin superfamily (IgSF) proteins, have been implicated in the recognition between synaptic partners (de Wit and Ghosh, 2016; Sanes and Zipursky, 2020). Among the IgSF, the Dpr and DIP subfamilies have recently emerged as candidates for synaptic recognition molecules.

Dpr/DIP interactions instruct MN-muscle synaptic recognition in *Drosophila*

Dprs belong to a subfamily of Ig proteins that contain two Ig domains (Nakamura et al., 2002). A high-throughput oligomerization–based assay to assess protein–protein interactions identified interactions between the Dprs and another previously uncharacterized IgSF subfamily, which was later named the Dpr–interactions proteins (DIPs; Ozkan et al., 2013; Carrillo et al., 2015). Follow-up studies revealed the full interactome between the 21 Dprs and 11 DIPs (Carrillo et al., 2015) and the binding affinities of each Dpr–DIP interacting pair and found a wide range of affinities (Cosmanescu et al., 2018), which may have functional significance (Xu et al., 2022). The Dpr–DIP interaction domain was mapped biochemically and through crystal structure analyses (Carrillo et al., 2015; Cosmanescu et al., 2018; Cheng et al., 2019).

The first report implicating Dprs and DIPs in circuit assembly showed that Dpr11 and an interacting partner, DIP-γ, are required for synaptic recognition between a subset of R7 photoreceptors and the Dm8 neurons in the medulla (Carrillo et al., 2015). The same interaction pair was required for Dm8 cell survival, and in the neuromuscular circuit, Dpr11 and DIP-γ mediate neuromuscular junction (NMJ) morphology (Carrillo et al., 2015). Other Dprs and DIPs have also been implicated in visual (Xu et al., 2018) and olfactory (Barish et al., 2018) circuit development. The Carrillo lab examined Dprs and DIPs in the embryonic/larval neuromuscular system and found that each MN expresses a unique subset of Dprs and DIPs (Y. Wang et al., 2022). Additionally, interactions between presynaptic

DIP-α and postsynaptic Dpr10 instruct MN→muscle synaptic recognition (Ashley et al., 2019). Overall, these studies show that Dprs and DIPs are multifunctional proteins in nervous system development. Learning how these cell surface proteins function combinatorially with each other and with other cell surface protein families will provide additional insight into motor circuit assembly and function.

A *C. elegans* MN undergoes Wnt-dependent neurite pruning

In addition to cell adhesion-based mechanisms that drive synaptic specificity, proper circuit assembly requires elimination of unnecessary neurites via pruning. During nervous system development, certain neuron types eliminate part of their neuronal processes in a stereotyped manner by a mechanism called developmental neurite pruning. Developmental neurite pruning is achieved by retraction or degeneration of neurites that are triggered by various extrinsic and intrinsic cues (Luo and O'Leary, 2005; Riccomagno and Kolodkin, 2015; Schuldiner and Yaron, 2015).

The cholinergic MN, PDB, located near the tail of *C. elegans* undergoes developmental neurite pruning controlled by LIN-44 (Wnt) and its receptor, LIN-17 (Frizzled). During development, PDB extends a single neurite toward the tail of *C. elegans*, where it forms transient neurites both posteriorly and anteriorly (Lu and Mizumoto, 2019). The posteriorly extended neurites located adjacent to the LIN-44-expressing hypodermal cells accumulate LIN-17, which promotes retraction, while the anterior neurite, which lacks LIN-17, continues its anterior extension and forms en passant NMJs onto the dorsal body wall muscles (Lu and Mizumoto, 2019). In the loss-of-function mutants of *lin-44* and *lin-17*, the posteriorly extended neurites remain due to failed retractions. While Wnt is a secreted morphogen that often functions as a gradient cue, the process of PDB pruning is not dependent on the gradient distribution of Wnt, as a membrane-tethered LIN-44 (neurotactin) is capable of inducing neurite pruning. Several studies have shown that the positions of NMJs of some MNs are determined by Wnt gradients (Klassen and Shen, 2007; Mizumoto and Shen, 2013). Interestingly, the position of PDB NMJs is defective in the animal with membrane-tethered LIN-44 (Lu and Mizumoto, 2019). This suggests that a neuron can utilize gradient-dependent and gradient-independent Wnt signaling in a context-dependent manner for shaping its proper structure and synaptic connections.

Upon Wnt binding, Frizzled elicits three distinct pathways; canonical Wnt→β-catenin pathway, Wnt→PCP (planar cell polarity) pathway, and Wnt→calcium pathway (Niehrs, 2012). The mechanisms by which LIN-44 and LIN-17 induce neurite pruning are unknown; however, none of the known components of the canonical Wnt-β-catenin and Wnt-PCP signaling pathways are involved in this process (Lu and Mizumoto, 2019), leaving the Wnt→calcium pathway as a potential candidate mechanism. Whether analogous pruning pathways are at play in the construction of mammalian spinal circuits remains to be determined.

Motor Circuits across Evolution

With the advent of new technologies to sequence, visualize, and perturb neurons across species, we can now study motor circuits in virtually any organism of choice. This gives us the newfound power to compare animal movement, and its underlying spinal and brain circuits, with unprecedented molecular and anatomical resolution (Wilson and Sweeney, 2023), enabling us to ask whether core conserved elements exist that span evolutionary time and, if so, how these elements may vary in their architecture with differences in movement repertoire.

Across vertebrates, such differences can be striking and include wide variation in (1) movement, and even the organization of the body, along the rostrocaudal axis with some organisms exhibiting highly coordinated forelimb movement (Iwaniuk and Whishaw, 2000) and others, no limbs at all (Jung and Dasen, 2015); (2) posture with some organisms completely upright, others not, and still others capable of transitioning smoothly between upright and prone locomotion (Ryczko et al., 2020); and (3) widely varying sensory, including both aquatic and terrestrial, environments that enable organisms to integrate their own environment-specific gravitational (Tuthill and Azim, 2018), temperature (Bokiniec et al., 2018), mechanical touch (Handler and Ginty, 2021), and noxious stimuli (Tracey, 2017).

Recent advances in single-cell sequencing have begun to reveal the building blocks of the central nervous system in new organisms (Arendt et al., 2019); viral tools have empowered us to look at their circuit connectivity (Nectow and Nestler, 2020; Jaeger et al., 2024), and CRISPR/Cas has even given us the ability to perform loss and gain of function in these new species. On top of this, machine learning algorithms to track behavior now allow us to score movement with improved precision in both space and time (Mathis et al., 2018; Pereira et al., 2022). With these tools, we can now begin to address if and how motor circuits scale for such vastly different forms of movement.

MNs provide a clear first readout for how circuits for different forms of movement are organized and offer a template for thinking about how the interneuron circuits that regulate MN firing might similarly vary. MNs most obviously differ across species in register to changes in muscle organization (Jung and Dasen, 2015). Anatomically, they are organized according to their projections in limbed vertebrates—those that innervate the same limb, hypaxial or axial region, are clustered into spatially and molecularly distinct columns and those that innervate the same muscle group into pools (Dasen and Jessell, 2009). Across species, there are also distinctions between neurons that are required for fast and slow movement—with these organized into distinct slow and fast types in both zebrafish (D'Elia et al., 2023; El Manira, 2023) and mammals (Schiaffino and Reggiani, 2011). Even with this substantial knowledge, however, we still do not fully understand the extent to which the molecular rules that dictate mammalian MN types apply also to lower vertebrates. In addition, we have gaps in our knowledge of how the composition of MN types, either molecularly, anatomically, or physiologically defined, varies across species. Large differences have been noted—amphibians, for example, lack gamma MNs—but the implications of these differences in movement remain to be explored.

Beyond MNs, our understanding of spinal and brain circuit scaling across species is even more incomplete. We do not know, even in mice, the full extent of inputs to each MN type—an obvious potential source of variation in MN output (Sengupta and Bagnall, 2023). When we consider spinal interneurons on their own, obvious similarities for specific classes have been found across vertebrates—with a correlate of the V0, V1, V2a, and V3 populations, for example, present in both mice and fish (Wilson and Sweeney, 2023). However, a comprehensive single-cell atlas of spinal interneuron diversity across vertebrate species is notably lacking—leaving us to postulate whether all 12 cardinal classes are present in both swimming and limbed species, and if so, how

much they differ in their fine-scale molecular, anatomical, and physiological properties.

To begin to build such a cross-species atlas, the Sweeney lab performed the first detailed and comprehensive comparison of motor and interneuron types between amphibians and mammals at both developing and adult stages. During development, the Sweeney lab found all 12 cardinal classes of interneurons in the frog, *Xenopus laevis*, as in the mouse. These 12 classes express the same core transcriptional determinants and exist in roughly the same proportions, demonstrating striking conservation between these two four-limbed species at this early stage. Only when we compare adult frog to adult mouse do we begin to see species divergence between spinal cord cell types. This divergence seems to be largely localized to the dorsal spinal cord and results from large differences in the expression of neuromodulatory genes, leading us to postulate that differences in sensory integration may be one of the most prominent species-specific features. Frogs and mice last shared a common ancestor 360 million years ago and exhibit vastly divergent movement patterns, with frogs exhibiting default synchronous movement, a nonweight-bearing posture, limited gaits, and very little dexterity, as compared with mice (Gordon et al., 2017), making conservation of ventral motor circuits at both stages quite remarkable.

One might also postulate that the spinal cord is remarkably similar across species, and in fact, it is the brain-to-spinal cord connectivity that has changed. Substantial evidence supports that descending input, especially corticospinal, is indeed one of the major evolutionary innovations (Olivares-Moreno et al., 2021). How does descending input vary across species? Can similarities be found in the organization of motor control regions in the brain, and how extensive are the differences? Where in the spinal cord is this input integrated?

One can also take a sensory-centric perspective—poising that it is the integration of sensory input that is the most species-specific feature and thus likely the most variant. Clear differences in sensation are immediately apparent—some organisms have hairy skin and others not, for example. This leads to the obvious question of how these sensory signals are transmitted and integrated differentially within the dorsal spinal cord. Do new sensory modalities require new dorsal cell types? The challenge, and significant opportunity, for motor neuroscientists moving forward is to harness the new technical approaches to parse cell-type diversity at a molecular, anatomical, and physiological level in new species and functionally link differences in circuits with differences in movement.

Closing Remarks

Despite recent progress in defining the mechanisms that dictate the development and function of motor circuits, several open questions remain:

1. What are the molecular principles that safeguard the identity and maintenance of MNs and other motor circuit cell types during adulthood?
2. How is the generation of interneuron diversity and integration into distinct functional circuits controlled?
3. What is the logic that governs the exquisite synaptic specificity within motor circuits?
4. How have motor circuits been adapted during evolution to support distinct motor behaviors across species?

The implementation of recent technological advances, such as rabies monosynaptic tracing, connectomic datasets, single-cell RNA sequencing, and CRISPR/Cas9 gene editing, in diverse vertebrate and invertebrate model organisms will continue to drive progress toward answering these and other open questions.

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