Establishing the Molecular and Functional Diversity of Spinal Motoneurons

Jeremy S. Dasen

Abstract Spinal motoneurons are a remarkably diverse class of neurons responsible for facilitating a broad range of motor behaviors and autonomic functions. Studies of motoneuron differentiation have provided fundamental insights into the developmental mechanisms of neuronal diversification, and have illuminated principles of neural fate specification that operate throughout the central nervous system. Because of their relative anatomical simplicity and accessibility, motoneurons have provided a tractable model system to address multiple facets of neural development, including early patterning, neuronal migration, axon guidance, and synaptic specificity. Beyond their roles in providing direct communication between central circuits and muscle, recent studies have revealed that motoneuron subtype-specific programs also play important roles in determining the central connectivity and function of motor circuits. Cross-species comparative analyses have provided novel insights into how evolutionary changes in subtype specification programs may have contributed to adaptive changes in locomotor behaviors. This chapter focusses on the gene regulatory networks governing spinal motoneuron specification, and how studies of spinal motoneurons have informed our understanding of the basic mechanisms of neuronal specification and spinal circuit assembly.

Keywords Motoneuron · Development · Spinal circuit · Neural patterning · Transcription factor

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1 Introduction

Although making up only a small fraction of all the neurons in the vertebrate central nervous system, motoneurons play crucial roles in facilitating nearly all aspects of animal behavior. Motoneurons within the spinal cord and brainstem are responsible for controlling basic motor functions including walking, breathing, posture, and balance. To accomplish their specific functions, motoneurons acquire specific features during embryonic development, including subtype-specific gene expression profiles, peripheral target specificity, and central connectivity with local and descending premotor networks. Compared to other neuronal classes, motoneurons have been relatively accessible to explore basic mechanisms of neuronal diversification and synaptic specificity. This is in part because the cell bodies of motoneurons targeting a specific peripheral target are organized into anatomically distinct columnar and pool groups. This relatively simple organization has enabled exploration in determining the relationship between the molecular profile and synaptic specificity of specific motoneuron subtypes.

In this chapter I describe studies that have illuminated the genetic programs governing vertebrate spinal motoneuron differentiation, focusing on the operation of gene regulatory networks that contribute to motoneuron subtype diversity, central organization, and connectivity. I will focus predominantly on the role of transcription factors (TFs), as they are primary targets of early signaling that pattern the neural tube, and play key roles in specifying both class- and subtype-specific features of motoneurons. I will describe some of the key targets of transcription factors that determine motoneuron central organization and peripheral target specificity. The evolutionary origins of motoneurons subtypes will be discussed, as well as plausible mechanisms through which these programs have been modified in vertebrates that have adapted specialized locomotor strategies. The role of motoneuron subtype identity in circuit assembly will be explored, particularly in relation to the local connections established between motoneurons, sensory neurons, and spinal interneurons. The overall theme is to highlight how studies on a single neuronal class has enabled multi-scale analyses of the development and function of motor circuits.

2 Anatomical and Functional Diversity of Spinal Motoneurons

At the time of their birth, all motoneurons possess a set of core features that distinguish them from other neuronal classes, but subsequently diversify into dozens of molecularly and anatomically specialized subtypes. Motoneurons subtypes can be differentiated on the basis of their anatomical position, functional properties, specificity of their connections, and molecular profiles. Historically, retrograde labeling of motoneuron through tracer injection into different muscle groups have allowed

characterization subtype identities on the basis of their organization within the spinal cord (Jessell et al. 2011; Landmesser 2001). These studies revealed that motoneurons targeting the same or related groups of peripheral targets are organized centrally into discrete clusters termed motor columns and motor pools (Fig. 1). Groups of motoneurons that target the same peripheral target can be further differentiated on the type of muscle fiber they innervate (i.e. fast versus slow, alpha versus gamma).

A major organizational feature of spinal motoneurons is their clustering into columns longitudinally arrayed along the rostrocaudal (head to tail) axis (Landmesser 1978b; Romanes 1941). Neurons within a column innervate a common set of target tissues, which includes both somatic muscles and visceral ganglia (Fig. 1a). Motoneurons targeting limb muscles are referred to as lateral motor column (LMC) neurons, indicating their position relative to medial motor column neuron (MMC) neurons, which target dorsal axial muscle. LMC neurons are generated specifically in brachial and lumbar levels of the spinal cord and innervate forelimb and hindlimb muscles, respectively, while MMC neurons are present at each segmental level. At thoracic levels visceral preganglionic column (PGC) neurons, which derive from the same precursors as somatic motoneurons, innervate sympathetic ganglia (Fig. 1c) (Prasad and Hollyday 1991). Visceral neurons sharing genetic signatures with thoracic PGC neurons are also present in sacral segments (Espinosa-Medina et al. 2016). Motoneurons of the hypaxial motor column (HMC) innervate intercostal and abdominal wall muscles, and are found predominantly in thoracic segments (Gutman et al. 1993; Prasad and Hollyday 1991). In mammals, phrenic motoneurons (PMC) are generated at rostral cervical levels and targets the diaphragm muscle. Each of these columnar groups form within the spinal cord shortly after motoneurons are generated (between E10.5 and E12.5 in mouse), and this basic organizational pattern persists into adulthood.

Additional layers of somatotopic organization of motoneurons are present within a single motor column (Fig. 1b). At brachial and lumbar levels, the LMC segregates into two molecularly and anatomically distinct divisional subtypes: a medial division (LMC_m) which contains motoneurons that project axons ventrally in the limb, and a lateral division (LMC_l) that projects dorsally (Landmesser 1978a; Tosney and Landmesser 1985a, b). Dorsally projecting LMC_l axons typically innervate limb extensor muscles, whereas LMC_m axons innervate flexor muscles. The organization of LMC neurons into columnar and divisional groups is present in all tetrapod classes that have been examined, as well as some species of cartilaginous fish (Jung et al. 2014, 2018).

A third level of motoneuron organization is evident in the diversification of LMC neurons into motor pools (Fig. 1d, e). A motor pool is defined as the population of motoneurons that innervates a single muscle (Hollyday et al. 1977; Landmesser 1978b; Romanes 1942). Motor pools occupy distinct positions within the spinal cord, and are invariant among animals of the same species (Hollyday and Jacobson 1990; Landmesser 1978b). While both brachial and lumbar LMC neurons share a common columnar and divisional organization, the motor pools of these two populations are distinct, reflecting differences in the anatomy of forelimb and hindlimb

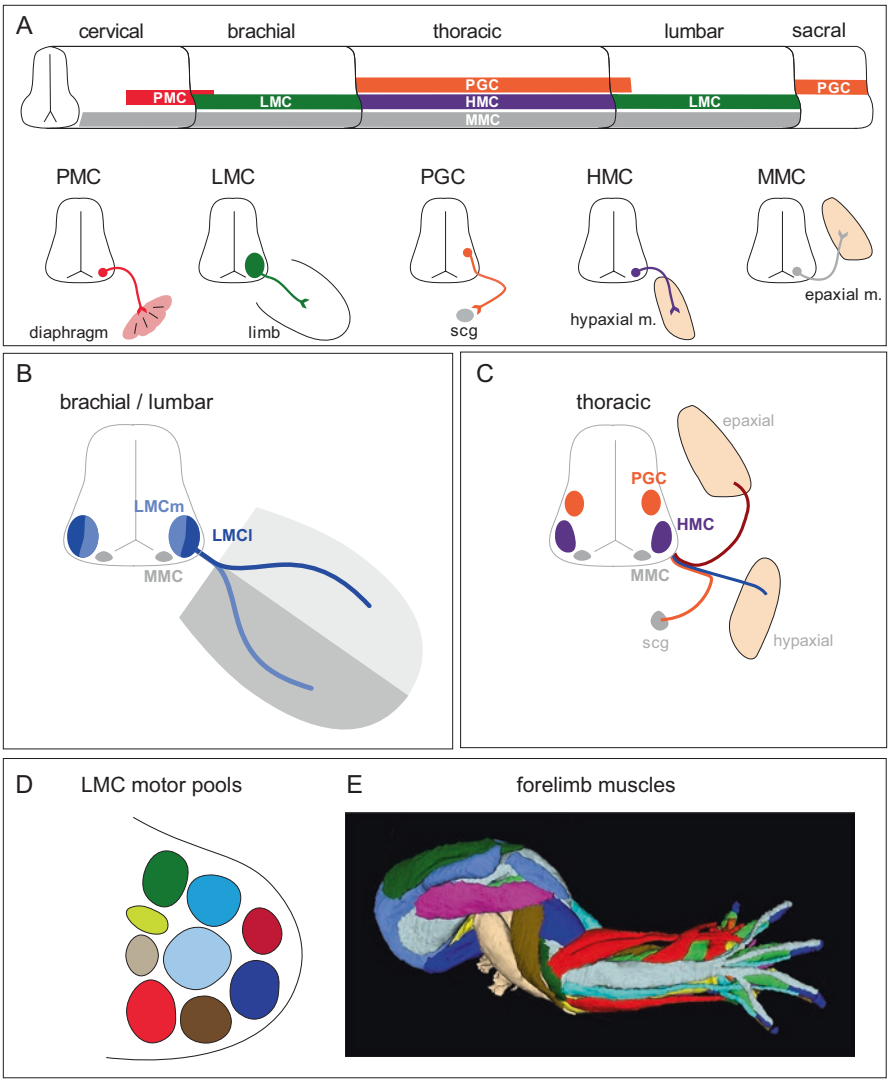

Fig. 1 Anatomical diversity of spinal motoneurons. (**a**) Organization of spinal motor columns and their peripheral targets. Phrenic motor column (PMC) neurons are found at cervical levels and target the diaphragm. Lateral motor column (LMC) neurons are generated at brachial and lumbar levels an innervate limb muscle. Preganglionic motor column (PGC) neurons are located in thoracic and sacral segments and target sympathetic chain ganglia (scg). Hypaxial motor column (HMC) neurons target ventral hypaxial muscle (e.g. intercostal and abdominal muscles). Medial motor column (MMC) neurons target dorsal epaxial muscles. (**b**) Motoneuron columnar organization at limb levels. Divisional identities of LMC neurons and initial trajectories are shown. LMC_l neurons target the dorsal limb compartment and LMC_m neurons project ventrally. (**c**) Organization of motor columns in thoracic segments. Motor axon projections of PGC, HMC, and MMC neurons are shown. PGC neurons are also observed in cervical, rostral lumbar and at lumbar segment L6. (**d**) Motor pool organization. Each motor pool targets a specific limb muscle. (**e**) Forelimb muscles of an E12.5 mouse embryo. (Credit: Muscles and tendons of mouse forelimb. NIMR, MRC. Attribution-NonCommercial 4.0 International (CC BY-NC 4.0))

muscles (Hollyday 1980; Hollyday and Jacobson 1990). Nevertheless, motor pools of both brachial and lumbar LMC neurons are somatotopically organized. At both segmental levels, motor pools positioned rostrally in the LMC typically innervate rostral and proximal limb muscles while caudal motor pools project to more caudal and distal muscles (Catela et al. 2016; Hollyday and Jacobson 1990; Landmesser 1978b; Vanderhorst and Holstege 1997).

Why motoneurons organize into defined columnar and pool clusters is not well understood. As discuss later, one argument in the formation of motoneuron topographic maps is to facilitate connections between motoneuron subtypes with premotor interneurons and sensory neurons (Jessell et al. 2011). Motoneuron columnar and pool organization may also facilitate the coordinated rhythmic firing of motoneurons, through gap junctions that are transiently formed during embryogenesis (Chang et al. 1999; Fulton et al. 1980). This rhythmic bursting activity of motoneurons has been shown to be important during the selection of peripheral trajectories by motor axons (Hanson and Landmesser 2004, 2006)

Additional layers of motoneuron diversification are also present within a single motor pool (reviewed in Kanning et al. 2010). Motoneurons targeting skeletal muscle can synapse on either extrafusal fibers (alpha-motoneuron), intrafusal fibers (gamma-motoneuron), or both (beta-motoneuron). Alpha motoneurons innervate extrafusal muscle fibers, have large cell bodies, and are the main drivers of muscle contraction. Gamma motoneurons innervate intrafusal fibers associated with muscle spindles, while beta-motoneuron target both intrafusal and extrafusal fibers. Alpha-motoneurons can be further classified based on whether they innervate fast-fatigable, fast fatigue-resistant, or slow-fatigable muscle fiber types. The appearance of these functional characteristics of motoneurons appears to emerge in later development, and multiple functional subtypes can be present within a single motoneuron pool. Thus, while motoneurons represent only a small fraction of neurons within the CNS, they display remarkable degree of anatomical and functional diversity. The diversification of motoneurons is governed by genetic programs deployed during embryonic development.

3 Specification of Spinal Motoneuron Class Identity

Like many neuronal classes, motoneurons acquire their fates as a consequence of developmental programs operating during the early stages of embryonic development. Shortly after neural induction, when ectoderm acquires a neuronal fate, the neural plate closes to form the neural tube. Gradients of secreted signaling molecules act on progenitors within the neural tube to specify positional fates along both the dorsoventral and rostrocaudal axes. Along the dorsoventral axis neural progenitors acquire unique molecular identities through the actions of multiple secreted patterning molecules (morphogens). Sonic hedgehog (Shh) is secreted ventrally from the notochord and floor plate, while bone morphogenetic protein (BMP) and wingless (Wnt) signaling originates from the dorsal roof plate and surface ectoderm

(Fig. 2a) (Jessell 2000; Sagner and Briscoe 2019). Graded Shh signaling is essential for the specification of motoneuron progenitors, as well as multiple classes of ventral spinal interneurons. RA from the paraxial mesoderm, and fibroblast growth factors (FGFs) from the caudal mesoderm and tail bud also contribute to the specification of motoneuron progenitors (Novitch et al. 2003). These spatially-graded morphogens establish unique populations of progenitors in the ventral spinal cord through inducing specific patterns of transcription factor expression (Fig. 2a). Molecularly distinct progenitor domains give to a single or multiple classes of postmitotic neurons, including four interneuron classes (termed V0, V1, V2, V3) and spinal motoneurons.

Shh acts via the Gli (glioma-associated oncogene) family of transcription factors to regulate the expression profile of two classes of homeodomain TFs, termed Class I and Class II (Shirasaki and Pfaff 2002). Class II proteins are induced in progenitors by differing levels of Shh, while Class I TFs are repressed by Shh and expressed more dorsally (Briscoe et al. 2000). Gradients of Shh along the dorsoverntal axis are

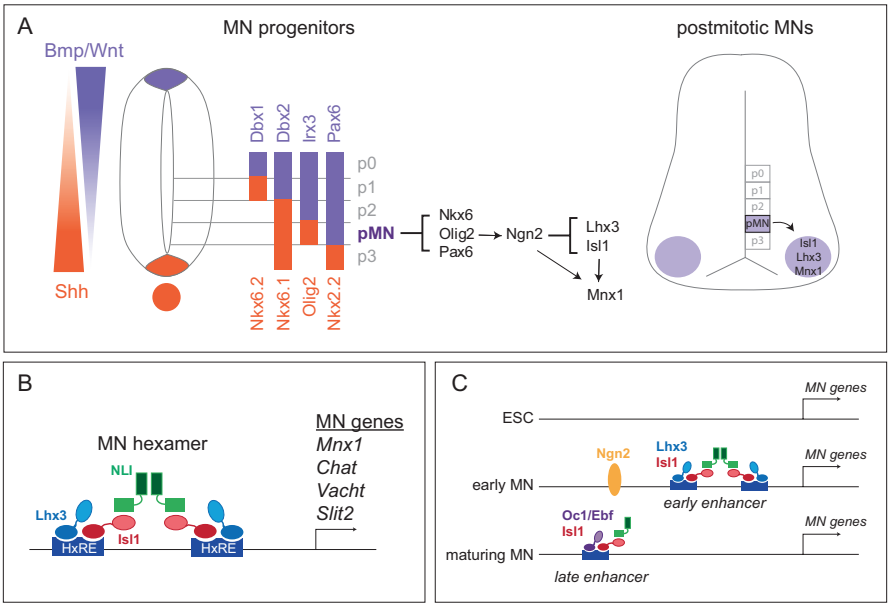

Fig. 2 Specification of core molecular feature of spinal motoneurons. (**a**) Early patterning of neural tube progenitors and motoneuron specification. BMP/Wnt and Shh gradients induce transcription factors in the neural tube. Motoneuron progenitors (pMN) and four ventral interneuron classes (p0, p1, p2, and p3) are shown. Olig2 promotes maintained expression of Neurogenin2 (Ngn2) which coordinately regulates motoneuron gene expression with Isl1 and Lhx3. Postmitotic motoneurons initially express Isl1, Lhx3, and Mnx1. (**b**) Transcriptional regulation of core motoneuron (MN) genes by Isl1, Lhx3, and NLI. Isl1, Lhx3 and NLI bind to hexamer response elements (HxRE) to regulate core motoneuron genes. (**c**) Enhancer switching during motoneuron maturation. Early motoneuron genes are coordinately regulated by Ngn2 and Isl1/Lhx3, later switching to enhancers containing Isl1/Oc or Isl1/Ebf motifs

interpreted by progenitors, in part, through spatial differences in the level of Gli activator and Gli repressor activity (Ribes and Briscoe 2009). In the absence of Shh, Gli repressor activity dominates, while Shh promotes ventral fates through promoting Gli activator function.

The initial patterns of Class I and Class II proteins are further fine-tuned and maintained through selective cross-repressive interaction between pairs of transcription factors (Balaskas et al. 2012). A consequence of these intrinsic transcriptional repressive networks is to establish sharp boundaries between progenitor domains and ensure the production of unique classes of postmitotic neurons (Fig. 2a). Spinal motoneuron progenitors (pMNs) are defined by the domain of Olig2, Pax6, and Nkx6.1 co-expression. Because cross-repressive interactions are critical in establishing the boundaries between progenitor domains, loss of one transcription factor often causes factors expressed in adjacent domains to become derepressed, leading to a switch in neuronal class fates (Ericson et al. 1997; Lanuza et al. 2004; Vallstedt et al. 2001). For example, mutation in the *Olig2* gene in mice leads to a loss of spinal motoneurons and a ventral expansion of V2 interneurons (Zhou and Anderson 2002).

Transcription factors expressed by progenitors play a critical role in setting up the regulatory landscape necessary for the emergence of general features of postmitotic motoneurons. Olig2 promotes expression of Neurogenin2 (Ngn2), and both Olig2 and Ngn2 operate to promote distinct aspects of motoneuron maturation (Mizuguchi et al. 2001; Novitch et al. 2001). Olig2 functions predominantly as a transcriptional repressor, where it establishes the pMN boundary. Olig2 also indirectly upregulates Ngn2 by repressing a repressor of *Ngn2* gene expression (Sagner et al. 2018). Ngn2 interacts with retinoic acid receptors (RARs) and histone modifying complexes to establish the chromatin environment critical for activation of panneuronal as well as motoneuron-restricted genes (Lee et al. 2009). Ngn2 also synergizes with the postmitotic motoneuron determinants Lhx3 and Isl1 to promote expression of motoneuron-restricted genes, including the transcription factor Mnx1 (also known as Hb9) (Lee and Pfaff 2003; Ma et al. 2008). Olig2 can counteract the function of Ngn2 in promoting motoneuron differentiation, suggesting the relative balance between Olig2 and Ngn2 expression serves as a gate for timing the activation of motoneuron-specific gene expression (Lee et al. 2005).

Like spinal progenitors, postmitotic neuronal classes can be defined by the specific transcription factors they express and can be further categorized on the basis of their connectivity pattern, neurotransmitter systems, and intrinsic physiological properties (Jessell 2000; Shirasaki and Pfaff 2002). Spinal motoneurons express a set of common early postmitotic determinants (Mnx1, Isl1, and Lhx3), extend axons outside the CNS, are cholinergic, and co-release glutamate (Mentis et al. 2005; Nishimaru et al. 2005). Lhx3, Isl1, Mnx1 play instructive roles in motoneuron specification, as misexpression of these factors, either alone or combination, can direct dorsal spinal interneurons to a motoneuron fate (Tanabe et al. 1998; Thaler et al. 2002). Genetic analyses in mice indicate that Lhx3 (and its paralog Lhx4), Isl1 (and Isl2), and Mnx1 are all essential for multiple aspects of motoneuron differentiation and peripheral connectivity (Arber et al. 1999; Liang et al. 2011; Sharma et al.

1998; Thaler et al. 1999, 2004). Isl1 and Lhx3 bind to the Lim-domain cofactor Lbd1 to promote spinal motoneuron identity by forming a hexameric complex consisting of two Lhx3:Isl1 dimers and NLI proteins (Fig. 2b) (Lee and Pfaff 2003). The interaction of these factors also prevents motoneurons from expressing genes associated with alternate spinal interneuron fates (Lee et al. 2008).

Because motoneurons represent only a small fraction of all the neurons in the spinal cord, it has historically been challenging to use biochemical approaches to determine the sets of genes that are directly regulated by motoneuron-restricted TFs. The ability to direct embryonic stem (ES) cells to a motoneuron fate has been an invaluable tool to determine the molecular mechanisms by which TFs regulate gene expression. ES cells can be differentiated to spinal motoneurons through treating with combinations of Shh agonist and RA, or through direct lineage programming by inducible expression of Ngn2, Lhx3, and Isl1 (Lee et al. 2012; Mazzoni et al. 2013a; Wichterle et al. 2002). Using these approaches, it has been demonstrated that the Lhx3-Isl1 complex directly binds and regulates a battery of genes associated with core motoneuron features, including other class-restricted transcription factors (i.e. Mnx1), cholinergic synthesis pathway genes, axon guidance receptors, and adhesion molecules (Fig. 2b).

Although the core TFs Isl1, Lhx3, and Mnx1 are expressed by all early born spinal motoneurons, they subsequently become restricted to distinct columnar, divisional, and pool subtypes. For example, while Lhx3 is required for the differentiation of all spinal motoneurons, it is subsequently restricted to MMC neurons which innervate dorsal axial muscle (Sharma et al. 1998). Thus, factors that regulate genes essential for motoneuron class identity are only transiently expressed, raising the question of how these programs are maintained after differentiation. Studies in ES-derived motoneurons indicate that gene enhancers occupied by Lhx3 and Isl1 are only transiently engaged during differentiation, subsequently switching to enhancers containing binding sites for Isl1, Onecut, and Ebf1 TFs (Rhee et al. 2016; Velasco et al. 2017). Maintenance of core motoneuron features therefore appears to depend on the switch from early enhancers bound by Lhx3-Isl1 to distinct enhancers that maintain expression in postmitotic motoneurons (Fig. 2c).

4 Early Patterning of Spinal Motoneurons Along the Rostrocaudal Axis

While all motoneurons share certain features, such as expression of genes encoding cholinergic synthesis and release pathways, they diversify into hundreds of subtypes. Motoneuron subtype identities have been traditionally defined by the specificity of their peripheral connections with muscle (Dasen and Jessell 2009; Landmesser 2001). Thus, efforts to define the developmental programs contributing to motoneuron subtype diversity have largely focused on the molecular profiles of neurons targeting specific muscle groups. The acquisition of muscle-specific fates

relies on patterning along the rostrocaudal axis. In addition to their early roles in establishing motoneuron progenitors, RA and FGFs determine the rostrocaudal positional identities of spinal progenitors (Bel-Vialar et al. 2002; Liu et al. 2001). RA and FGFs form opposing concentration gradients, where RA secreted from the paraxial mesoderm and somites acts as a rostralizing signal, while FGFs patterns more caudal levels (Fig. 3a).

RA and FGFs confer rostrocaudal positional identities to motoneurons and other neuronal classes through controlling the temporal and spatial expression of *Hox* family transcription factors (Bel-Vialar et al. 2002; Dasen et al. 2003; Liu et al. 2001). *Hox* genes are a large family of chromosomally arrayed genes comprising 39 genes encoded by 4 clusters (*HoxA*, *HoxB*, *HoxC*, and *HoxD*), most of which are expressed in the spinal cord and hindbrain (Parker and Krumlauf 2020; Philippidou and Dasen 2013). In general, *Hox1-Hox5* paralog group genes are expressed in the hindbrain while *Hox4-Hox13* genes are detected in the spinal cord. Unlike the sequential expression of different sets of TFs in motoneuron progenitors (Olig2, Pax6, Nkx6) and early postmitotic motoneurons (Mnx1, Lhx3, Isl1), *Hox* genes are expressed during both phases of motoneuron differentiation.

Expression of individual *Hox* genes directly correlates with its position within a chromosomal cluster, a principle termed spatial colinearity (Kmita and Duboule 2003). *Hox* genes positioned at the 3′ end of a cluster are expressed at rostral levels, while 5′ genes are expressed at more caudal regions of the neuroaxis (Fig. 3a). The initiation of *Hox* gene expression occurs during axis extension, as stem cell-like populations emerge from the node (the organizer for gastrulation in the vertebrate embryo), and generate neuronal and non-neuronal progenitors. This early rostrocaudal patterning step appears to be initiated prior to neurogenesis (Metzis et al. 2018). Growth of the tail bud is associated with the progressive removal of repressive chromatin marks from *Hox* loci, and the appearance of chromatin marks indicative of gene activation (Soshnikova and Duboule 2009).

RA has an important role in patterning *Hox* expression in the spinal cord where it regulates expression of genes associated with cervical/brachial levels (*Hox4-Hox6* genes), in conjunction with other signaling systems (Liu et al. 2001). RA acts through retinoic acid receptors (RARs) to directly activate *Hox1-Hox5* paralogs (Mazzoni et al. 2013b). FGF signaling has a prominent role in establishing the patterns of *Hox4-Hox10* gene expression in the spinal cord and increasing levels of FGF induces *Hox* genes with a progressively more posterior character (Liu et al. 2001; Peljto et al. 2010). Artificially increasing FGF signaling in vivo induces a rostral shift of *Hox* expression and transforms the identities of brachial motoneuron subtypes to a thoracic fate (Bel-Vialar et al. 2002; Dasen et al. 2003, 2005). The effects of FGF signaling in the neural tube are mediated through Cdx homeodomain proteins, as FGF can induce *Cdx* expression, and Cdx proteins can directly activate expression of caudal *Hox* genes (Bel-Vialar et al. 2002; Mazzoni et al. 2013b). FGFs also works in concert with other signaling systems to orchestrate patterns of Hox expression in the spinal cord. At rostral levels FGF acts with RA to establish expression of *Hox6-Hox8* genes in brachial motoneurons. At more posterior levels, FGFs functions with secreted Growth differentiation factor 11 (Gdf11) to initiate

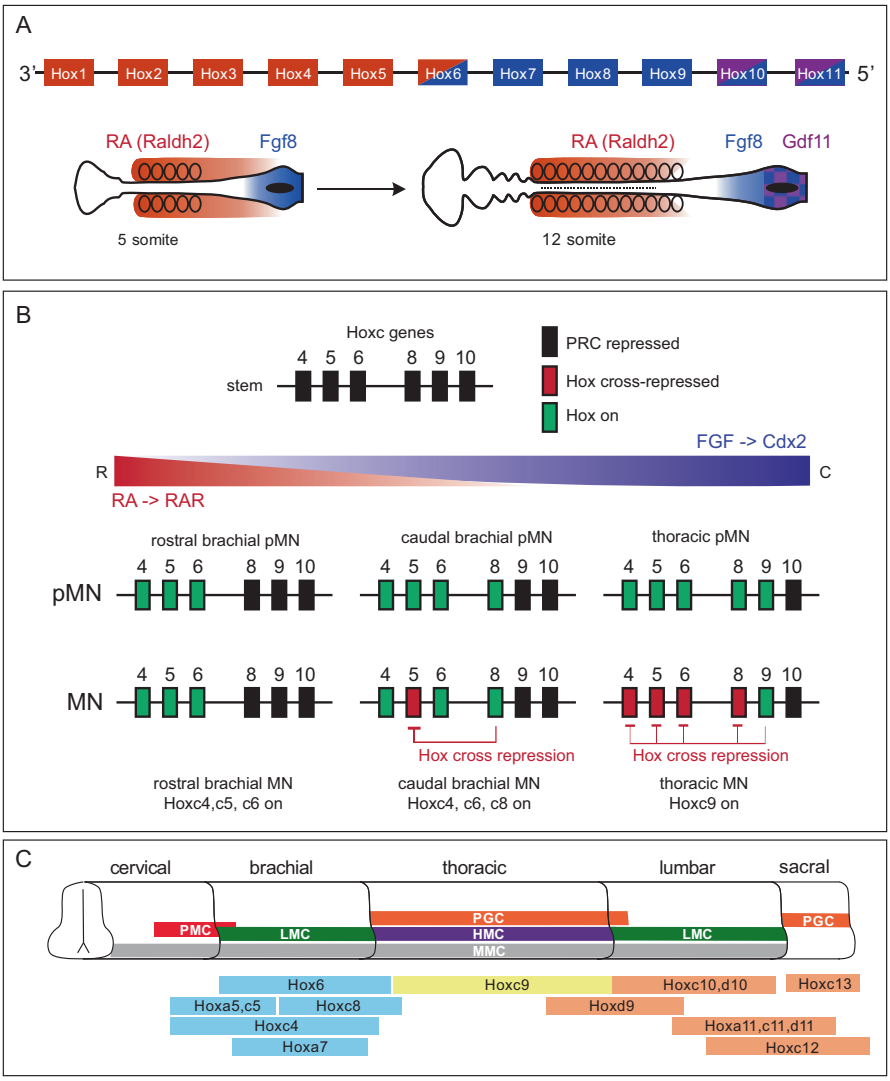

Fig. 3 Regulation of *Hox* gene expression along the rostrocaudal axis. (**a**) Patterning of *Hox* gene expression by RA, FGF, and Gdf11. Chromosomally arrayed *Hox* genes are activated by gradients of signaling molecules. Color coding of *Hox* genes denotes paralog groups regulated by indicated morphogens. RA induces *Hox1-Hox5* genes, FGF *Hox6-Hox9*, and Gdf11/FGF8 *Hox10-Hox11* genes. (**b**) Regulation of *Hox* gene expression by Polycomb repressive complexes (PRCs) and Hox cross-repression in brachial and thoracic segments. A subset of *HoxC* cluster genes is shown, which are silenced in stem cells by PRCs (indicated in black). In progenitors, RA (through RAR) and FGFs (through Cdx2) remove PRC marks sequentially from *Hox* genes, activating their expression (indicated in green). In early postmitotic motoneurons, Hox cross-repressive interactions establish caudal *Hox* boundaries (indicated in red). (**c**) Summary of *Hox* expression in motoneurons. Hox patterns and lumbar and sacral segments are based on mRNA expression and have not been confirmed with protein analyses

expression of *Hox10* genes which define lumbar fates (Liu et al. 2001). Wnt signaling also functions in rostrocaudal patterning and modulates the responsiveness of progenitors to RA and FGF (Nordstrom et al. 2006). Subsequent to these early patterning events, the rostral boundaries of *Hox* gene expression are maintained through differentiation by the action of the Polycomb group family of repressor proteins (Fig. 3b) (Golden and Dasen 2012; Mazzoni et al. 2013b; Narendra et al. 2015).

The pattern of *Hox* genes induced by morphogens in spinal progenitors is characterized by specific rostral boundaries, with caudal boundaries that often extend to the tail bud. Thus, there is initially extensive overlap in *Hox* mRNA expression in caudal neural progenitors. By the time of motoneuron differentiation posterior boundaries become apparent. These caudal boundaries are established through cross-repressive interaction between *Hox* genes (Fig. 3b) (Dasen et al. 2003, 2005). The molecular mechanisms mediating Hox cross-repressive interactions have been studied in detail for the Hoxc9 protein, which is expressed by thoracic motoneurons where it represses brachial *Hox4-Hox8* genes (Jung et al. 2010). In *Hoxc9* mutants, *Hox4-Hox8* paralog genes are derepressed at thoracic levels leading a transformation of thoracic motoneurons to a brachial LMC fate (Jung et al. 2010). In addition, within a specific segmental level, cross-repressive interactions amongst *Hox* genes contribute to the diversification of motor pools (Catela et al. 2016; Dasen et al. 2005).

Each of the segmentally-restricted motoneuron columnar subtypes can be defined by a specific pattern of postmitotic Hox expression (Fig. 3c). At cervical levels, Hox5 proteins are expressed by phrenic motor column neurons (Philippidou et al. 2012). Hox6 and Hox10 proteins mark brachial (forelimb-innervating) and lumbar (hindlimb) LMC neurons, respectively (Dasen et al. 2003, 2008; Lacombe et al. 2013; Rousso et al. 2008; Shah et al. 2004; Wu et al. 2008). Intervening limb-level LMC neurons, the *Hoxc9* gene is essential for the appearance of thoracic neuronal fates, including preganglionic column (PGC) neurons (Jung et al. 2010). Expression of an additional two dozen *Hox* genes within the LMC defines the molecular identity of motor pools (Dasen et al. 2005). The next section describes the roles Hox proteins in determining the molecular profiles and peripheral target specificity of motoneuron subtypes.

5 Developmental Mechanisms of Motoneuron Diversification

Patterning systems along the rostrocaudal axis act to define the expression profiles of *Hox* genes in motoneurons. While *Hox* gene expression is initiated in progenitors, they continue to be expressed by early postmitotic motoneurons, where they play important roles in generating segmentally-restricted subtypes. Although how dorsoventral and rostrocaudal patterning systems are integrated during development is poorly understood, it appears that Hox proteins operate in conjunction with early motoneuron determinants (e.g. Mnx1, Isl1) to generate segment-specific subtypes. In parallel with these programs, Hox-independent pathways are also deployed to specify the identities of motoneuron subtypes innervating axial muscle.

Hox Genes and the Specification of LMC Neurons and Divisional Subtypes

Because of their critical roles in facilitating limb movement, the specification of LMC neurons has been thoroughly examined, both in terms of the genetic programs that specify their subtype identities and mechanisms of muscle-target specificity (Fig. 4a). As with other neuronal classes, the diversification of LMC neurons involves a hierarchical program that progressively refines the molecular profiles of motoneurons and shapes the specificity of limb innervation. Early in development, Hox transcription factors ensure that LMC neurons are generated in register with the developing limb buds. As LMC neurons further mature, a combination of both motoneuron-intrinsic and limb-derived cues operate to generate the diversity of

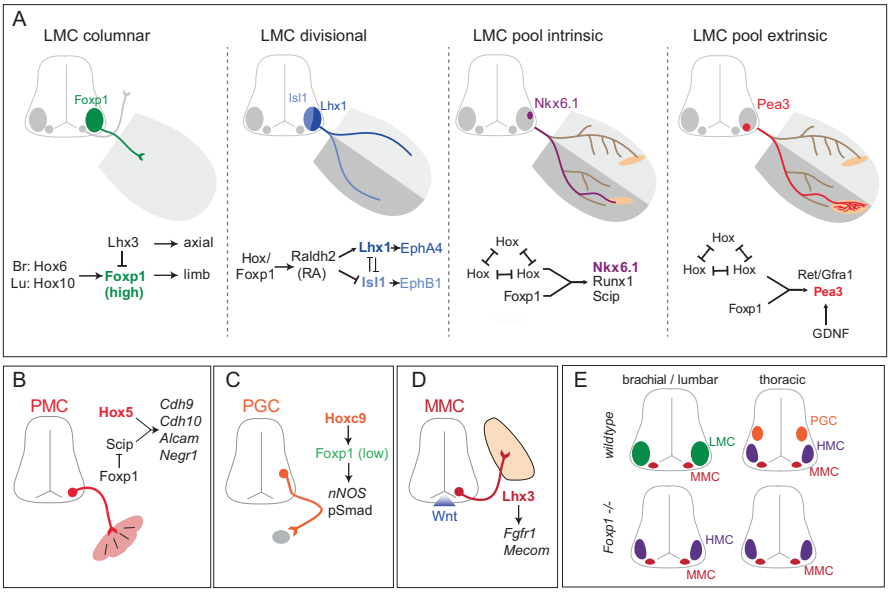

Fig. 4 Diversification of spinal motoneuron subtype identities. (**a**) Transcriptional regulation of LMC diversification. Hox proteins expressed at limb levels promote high expression of Foxp1. Brachial (Br) LMC neurons express *Hox6* genes, while lumbar (Lu) LMC neurons express *Hox10* genes. Foxp1 is required for Raldh2 expression, which contributes to the specification of LMC divisional identities. Isl1 and Lhx1 promote expression of Eph receptors within LMC divisions. Hox proteins specify motor pool fates by regulating expression of downstream transcription factors (e.g. Nkx6.1, Scip, Runx1). Limb-derived cues are also required to induce other pool-specific factors such as Pea3 and Etv1. (**b**) PMC development. Hox5 proteins act in conjunction with Scip to promote PMC-restricted gene expression. Foxp1 represses Scip expression. (**c**) PGC specification. Hoxc9 promotes a low level of Foxp1 expression. Foxp1 is required for the appearance of markers for PGC fate, including nNOS, and pSmad1/5/8. (**d**) MMC neuron development. Wnt signaling promotes maintained expression of Lhx3 in MMC neurons. Lhx3 regulates expression of Fgfr1 and the MMC-restricted marker Mecom. (**e**) Consequences of *Foxp1* mutation. In *Foxp1* mutants presumptive brachial/lumbar LMC and thoracic PGC neurons acquire an HMC identity

LMC subtypes necessary to innervate the dozens of muscles present in a typical tetrapod limb.

Hox proteins confer subtype-specific features of motoneurons, in part, through regulating expression of other transcription factors. A key target of Hox proteins in spinal motoneurons is the transcription factor *Foxp1*, which is expressed by LMC neurons, and at reduced level by thoracic and sacral PGC neurons (Dasen et al. 2008; Espinosa-Medina et al. 2016; Rousso et al. 2008). The pattern of Foxp1 in motoneurons is determined by Hox proteins expressed at specific segmental levels. Hoxc6 and Hoxc10 promote high levels of Foxp1 in brachial and lumbar LMC neurons, respectively, while Hoxc9 sets low Foxp1 levels in thoracic PGC neurons. Misexpression studies in chick embryos indicate that Hoxc6 and Hoxd10 can convert thoracic motoneurons to an LMC fate, as measured by ectopic expression of high Foxp1 levels and other LMC molecular markers (Dasen et al. 2008). Hox proteins therefore appear to act in the early phases on LMC differentiation to induce expression of genes that regulate subsequent steps of diversification.

While expression of individual *Hox* genes correlates with the positioning brachial and lumbar LMC neurons, there is significant redundancy among *Hox* genes during LMC specification. Although *Hox6* genes are expressed by the majority of brachial LMC neurons, in mice lacking all *Hox6* paralog genes (*Hoxc6*, *Hoxa6,* and *Hoxb6*) LMC neurons are still generated, although in reduced numbers (Lacombe et al. 2013). Misexpression studies in chick indicate that multiple *Hox5-Hox8* paralogs can confer an LMC identity to thoracic motoneurons, suggesting LMC specification involves the activities of multiple redundant Hox inputs. Consistent with this idea, combined mutation of the *HoxA* and *HoxC* gene clusters abolishes the specification of brachial LMC neurons (Jung et al. 2014). Thus, the early columnar identity of LMC neurons is determined through redundant Hox inputs that regulate the pattern of Foxp1 expression.

Foxp1 is essential for specifying nearly all early molecular features of LMC neurons. Genetic depletion of *Foxp1* in mice does not preclude the expression of general motoneuron features, such as expression of Mnx1 and cholinergic synthesis pathway genes, or affect the pattern of Hox expression (Dasen et al. 2008; Rousso et al. 2008). In *Foxp1* mutants, prospective LMC neurons fail to express early embryonic markers of motoneuron columnar, divisional and pool identities. In the absence of Foxp1, presumptive LMC and PGC neurons express markers of ventrally-projecting HMC neurons (Fig. 4c). These observations suggest that the HMC subtype represents an ancestral population from which LMC neurons and other segmentally-restricted subtypes evolved.

Foxp1 is also required for the expression of the retinoic acid synthetic enzyme Raldh2, which is expressed by brachial and lumbar LMC neurons. Raldh2 expression in motoneurons creates a local neuronal source of RA, and is involved in specifying LMC divisional identities (Sockanathan and Jessell 1998). Neurons within the LMC differentiate into to a lateral division (LMC_l) which targets muscle in the dorsal limb compartment, and a medial division (LMC_m) that targets ventral muscles (Fig. 4a). Each of these divisional groups are characterized by selective expression of Lim homeodomain (HD) proteins: LMC_l neurons express Lhx1, while

LMC_m neurons express Isl1 (Tsuchida et al. 1994). Expression of Lhx1 in LMC_l neurons is regulated by local RA signaling from $Raldh2^+$ LMC neurons, as well as RA from the paraxial mesoderm, and is consolidated through cross-repressive interactions between Lhx1 and Isl1 (Ji et al. 2006; Kania et al. 2000; Sockanathan and Jessell 1998). The activities of Lim HD factors in LMC_l and LMC_m also play important roles in determining the selection of axonal trajectories within the limb. Lhx1 promotes the dorsal projection of LMC_l neurons through regulating expression of the guidance receptor EphA4, which repels axons from ephrin-expressing ventral limb mesenchyme (Eberhart et al. 2002; Kania and Jessell 2003). Lim HD protein mediated regulation of Eph receptor/ephrin interactions are also involved in determining the ventral projection of LMC_m axons (Luria et al. 2008).

Intrinsic Programs of Motoneuron Pool Specification

Despite the converging actions of multiple redundant *Hox* genes to specify LMC fate, individual *Hox* genes are required to diversify LMC neurons into motor pools targeting specific limb muscles. Of the 39 *Hox* genes present in mammalian genomes, approximately half are expressed by spinal motoneurons, with the majority being expressed in LMC neurons (Dasen et al. 2005; Lacombe et al. 2013). Brachial LMC neurons express *Hox4-Hox9* paralogs, while lumbar LMC neurons express *Hox9-Hox12* genes. The profile of Hox expression in LMC motor pools is established through repressive interactions between *Hox* genes in motoneurons, contributing to both their rostrocaudal and intrasegmental organization. For example, repressive interactions between Hoxc5 and Hoxc8 establish the boundary between rostral and caudal brachial LMC motor pools, while a network of interactions between Hox4-Hox9 paralogs shape motor pool intrasegmental diversity (Catela et al. 2016; Dasen et al. 2005; Mendelsohn et al. 2017).

As with the specification of motoneuron columnar identity, Hox proteins determine motor pool diversity through regulating expression of downstream transcription factors. For example, the ETS domain TF Pea3 (also known as Etv4), the Pou domain protein Scip (Pou3f1), and Nkx6.1 are all expressed by pools of LMC neurons targeting specific limb muscles. Expression of these pool-restricted factors requires specific combinations of Hox proteins. Both Hoxc8 and Hoxc6 are required for the specification of the brachial motor pools defined by Pea3 expression, while only Hoxc8 is required for expression of Scip. In both *Hoxc6* and *Hoxc8* mutants there is a depletion in Pea3+ motoneurons and a severe reduction in the arborization of the muscle normally targeted by this pool (Catela et al. 2016; Lacombe et al. 2013; Tiret et al. 1998). Analyses of mice lacking the motor pool restricted TF Nkx6.1, demonstrate that downstream targets of Hox proteins are critical for muscle target specificity (De Marco Garcia and Jessell 2008). The motor pool-restricted expression of *Pea3*, *Scip*, and *Nkx6.1* is also lost in *Foxp1* mutants, indicating that Hox factors work in conjunction with Foxp1 to specify the molecular identities of

motor pools. Foxp1 therefore acts both as a target and an accessory factor of Hox proteins to specify motoneuron pool fates.

Collectively, studies of *Hox* gene function indicate varying degrees of redundancy at the level of motoneuron columnar identity, but highly specific roles during motor pool specification. Multiple *Hox* genes promote the specification of LMC identity, through regulation of *Foxp1* expression, while individual *Hox* genes specify the molecular profiles and connectivity of motor pools. Because *Hox* expression is dynamic during the course of motoneuron pool differentiation, one possible purpose of *Hox* redundancy is to ensure LMC neurons maintain *Foxp1* expression in circumstances where individual *Hox* genes are downregulated. For example, in caudal cervical LMC neurons expression of Hoxc6 is downregulated in a subset of Foxp1^{+} LMC neurons, while expression of Hoxc8 is maintained.

Target-Dependent Regulation of Motoneuron Pool Identities

While early features motor pool identity are controlled through cell-intrinsic Hox networks, the maturation of motoneurons also depends on cues from peripheral targets. Expression of the ETS transcription factors Pea3 and Etv1 in LMC pools depends on neurotrophic signals provided by the limb mesenchyme and muscle (Haase et al. 2002; Lin et al. 1998). These signals appear to be permissive rather than instructive and not all motoneurons are competent to respond to neurotrophin signaling. In explants of spinal cord treated with glial-derived neurotrophic factor (GDNF), Pea3 is induced in a pattern approximating the normal distribution observed *in vivo* and is confined to the caudal LMC segments that normally express Pea3 (Haase et al. 2002). Ectopic expression of Hoxc8 in rostral LMC neurons can expand the domain of Pea3 expression (Dasen et al. 2005), suggesting that Hoxc8 activity defines the region in which LMC neurons are competent to respond to GDNF. Consistent with this model, in *Hoxc8* mutants motoneurons fail to fully activate Pea3 expression (Catela et al. 2016; Vermot et al. 2005). Hoxc8 promotes the ability of motoneurons to respond to GDNF by regulating expression of the Ret and Gfra1 receptors, which are required to transduce GDNF signaling (Catela et al. 2016).

These observations indicate that target-derived cues contribute to the programming of motor pool fates. Expression of the target-induced factor Pea3 is critical for multiple aspects of differentiation, including the clustering of motoneurons into pools, muscle-specific patterns of axonal innervation, and sensory-motor connectivity (Livet et al. 2002; Vrieseling and Arber 2006). Motor pool specification therefore appears to unfold in two main phases: an early Hox/Foxp1-dependent phase that confers aspects of motoneuron molecular identity involved in the selection of target muscle connectivity (Landmesser 2001; Milner and Landmesser 1999), and a later phase, that is associated with ETS gene expression and the clustering of motoneurons within the LMC (Livet et al. 2002; Price et al. 2002).

Development of Phrenic Motor Column (PMC) Neurons

Breathing is an essential motor behavior which relies on rhythmic activation of phrenic motor column (PMC) neurons. PMC neurons are unique to mammals and are located in rostral cervical segments of the spinal cord. As with other segmentally-restricted motoneuron subtypes, PMC neuron development relies on *Hox* function to deploy subtype-specific gene regulatory programs (Fig. 4b). Two Hox5 paralogs, Hoxa5 and Hoxc5 are expressed by PMC neurons, and in the absence of *Hox5* function in mice, animals perish at birth due to respiratory failure (Philippidou et al. 2012). In *Hox5* mutants, motor axons extend to the diaphragm, but fail to arborize within the muscle, and PMC neurons eventually are lost due to programmed cell death. Hox5 proteins appear to act in conjunction with another transcription factor, Scip, to deploy PMC-specific gene programs in motoneurons (Machado et al. 2014; Philippidou et al. 2012).

Studies of *Hox5* gene function in PMC development have also provided insights into the development of spinal respiratory circuits required for breathing. Hox5 proteins are required to establish the stereotypical dendritic organization of PMC neurons, and *Hox5* mutants are characterized by an increased crossing of dendrites to the contralateral spinal cord (Vagnozzi et al. 2020). Hox5 proteins also regulate expression of cadherins in PMC neurons, and loss of cadherin function leads to similar defects in dendritic morphology. *Hox5* mutants are characterized by marked reduction in the number of inhibitory inputs, possibly a consequence of changes in the molecular profiles or dendritic morphology of PMC neurons. This loss of inhibitory inputs onto PMC neurons likely contributes to the respiratory defects observed in mice with reduced *Hox5* function (Vagnozzi et al. 2020).

Development and Diversity of Preganglionic Column (PGC) Neurons

In addition to muscle-innervating somatic motoneurons, the spinal cord contains an additional class of neurons that derive from the same progenitor domain as motoneurons and target peripheral ganglia in the sympathetic nervous system (Fritzsch et al. 2017). Although not directly involved in skeletal muscle contraction, preganglionic column (PGC) neurons share multiple features with somatic motoneurons, including expression of Lim HD factors, cholinergic synthesis genes, and projection of axons outside the CNS. Spinal PGC neurons are located predominantly in thoracic, rostral lumbar, and sacral segments and innervate sympathetic chain ganglia. PGC neurons are born in the ventral spinal cord near somatic motoneurons, migrating to a dorsomedial position in chick (forming the "Column of Terni"), while in mouse they migrate to a mediolateral position. In both species, embryonic PGC neurons can be distinguished from somatic motoneurons by selective

phospho-Smad1/5/8 immunoreactivity. In mice, PGC neurons are also marked by expression of neuronal nitric oxidase synthase (nNOS).

Genetic studies indicate that like LMC neurons, PGC neuron molecular identities are determined by Hox transcription factor activity, which regulates the pattern of Foxp1 expression (Fig. 4c). Mutation in either *Hoxc9* of *Foxp1* leads to a loss of PGC marker expression, and sympathetic chain ganglia lack innervation (Dasen et al. 2008; Jung et al. 2010; Rousso et al. 2008). Hoxc9 functions to generate neurons that express low levels of Foxp1, and manipulations that reduce *Foxp1* levels in vivo can generate ectopic PGC neurons. For example, misexpression of Hoxc9 in brachial LMC neurons, reduces Foxp1 expression to low levels and converts LMC neurons to a PGC fate. Sacral PGC neurons also express *Foxp1* (Espinosa-Medina et al. 2016), and likely depend on sacral-level *Hox* genes for their specification.

While studies of visceral motoneuron specification have focused on a few known molecular markers, more recent studies have revealed that, like LMC neurons, PGC neurons are molecularly highly diverse. Single cell RNA sequencing studies on adult motoneurons have revealed up to 16 distinct subtypes, characterized by selective expression of neuropeptides and other neuromodulatory proteins (Alkaslasi et al. 2021; Blum et al. 2021). Interestingly, these studies also reveal that PGC neurons are not exclusive to thoracic and upper lumbar segments, but are also detected in cervical spinal cord.

Specification Hypaxial (HMC) and Medial Motor Column (MMC) Neurons

In contrast to the Hox-dependent programs that determine the identities of segmentally-restricted subtypes, motoneurons innervating axial muscles can be present throughout the spinal cord, and do not appear to directly rely on Hox protein function for their specification. Axial motoneurons can be broadly divided into two columnar subtypes, depending on whether they innervate dorsal epaxial or ventral hypaxial muscle. Dorsal epaxial muscles are associated with posture and balance, and are innervated by neurons in the medial motor column (MMC). Hypaxial muscles reside ventrally, include abdominal and intercostal muscles, and are innervated by HMC neurons. Similar to limb motoneurons, both MMC and HMC neurons also appear diversify into muscle-specific pools (Gutman et al. 1993; Smith and Hollyday 1983). Although the genetic programs that specify muscle-specific subtypes of axial motoneurons are not understood, recent studies have begun to define a few markers that may delineate MMC and HMC pools (Catela et al. 2019; Hanley et al. 2016).

MMC and HMC neurons can be distinguished by the absence of Foxp1 expression and maintained expression of factors involved in early motoneuron fate, such as Isl1, Lhx3, and Mnx1 (Dasen et al. 2008; Tsuchida et al. 1994). MMC neurons maintain expression of Lhx3, whereas HMC neurons, like most other columnar subtypes, downregulate Lhx3 as they differentiate. The maintained expression of

Lhx3 in MMC neurons appears to be regulated by Wnt signaling originating from the floorplate (Fig. 4d) (Agalliu et al. 2009). Elevation of Wnt signaling in vivo promotes the generation of MMC neurons at the expense of other motoneuron subtypes, while combined genetic removal of *Wnt* genes depletes MMC numbers. MMC neurons can also be defined expression of the Prdm family transcription factor Mecom, and Mecom expression is regulated by Lhx3 and Mnx1 (Hanley et al. 2016). Although Mecom is one of the few TFs selectively expressed by MMC neurons, its function is currently unknown.

Lhx3 is expressed by the precursors of all spinal motoneurons, and is required for the differentiation of most subtypes, confounding attempts to understand its specific function during MMC development (Sharma et al. 1998). Gain of function studies show that Lhx3 can convert Hox-dependent LMC and PGC neurons to an MMC fate and redirect motor axons dorsally to epaxial muscle. Lhx3 therefore has a dominant and instructive role in determining the identity and trajectories of MMC axons (Sharma et al. 2000). The projection of MMC neurons has been shown to depend on target-derived chemoattractant signaling emanating from a somite-derived structure, the dermomyotome (Tosney 1987, 1988). The dermamyotome expresses FGFs which act on the axons of MMC neurons, which selectively express FGF receptor 1 (Fgfr1) (Shirasaki et al. 2006). Mutation of *Ffgr1* in mice causes defects in the peripheral trajectory of MMC axons. In addition, misexpression of Lhx3 leads to ectopic expression of *Fgfr1* in non-MMC motoneurons and causes limb motor axons to gain sensitivity to FGFs (Shirasaki et al. 2006).

Studies in ES cell-derived motoneurons suggest that additional signaling pathways act in conjunction with Wnt signaling to promote MMC specification (Tan et al. 2016). Inhibition of Notch signaling in ES-cell derived motoneurons promotes the specification of HMC neurons at the expense of MMC neurons, suggesting that Wnt4/5 and Notch cooperate to specify MMC identity. These observations also raise the possibility that signaling between newly born motoneuron may play a role in subtype differentiation.

In contrast to MMC neurons, little is known about the developmental programs that specify HMC fate. Although trunk-level HMC neurons can be defined by expression of specific transcription factor combinations (Mnx1^{+}, Isl1/2^{+}, Lhx3^{-}, Foxp1^{-}), the intrinsic determinants of HMC identity are currently unknown. There are only a few known genetic manipulations that disrupt the specification of HMC neurons *in vivo*. For example, mutation of the *Hoxc9* gene, which is expressed broadly in thoracic segments, causes a derepression of multiple *Hox4-Hox8* genes and a conversion of presumptive HMC neurons to an LMC fate. By contrast, in *Foxp1* mutants the number of HMC expands throughout the spinal cord. It is possible that HMC neurons may represent a motoneuron ground state, through which instructive factors like Lhx3 and Hox proteins can specify subtype identities.

Establishing Motoneuron Functional Subtype Diversity

While studies of motoneuron diversification have largely focused on the developmental programs involved in establishing subtypes defined by their peripheral targets, motoneurons also acquire additional features related to the type of muscle fiber innervated (Kanning et al. 2010; Stifani 2014). Motoneurons can be classified as alpha, beta, or gamma depending on their innervation of intrafusal versus extrafusal fibers. Alpha motoneurons can also be classified as fast or slow, depending on the type muscle fiber a motoneuron innervates. Thus, even within a single motor pool, there are additional layers of diversification that contribute to motoneuron functional features.

Studies have revealed some of the pathways and molecular signatures that specify motoneuron functional subtype identities. Gamma motoneurons are marked by expression of *Wnt7a*, and expression of this marker depends on the presence of muscle spindles (Ashrafi et al. 2012). This observation suggests spindle-derived cues are involved in gamma motoneuron specification. The delta-like homolog Dlk-1 has been shown to promote the biophysical signatures of fast motoneurons, by activating expression of the potassium channel subunit Kcng4 (Muller et al. 2014). A slow muscle-fiber type specific cue also appears to promote the expression of molecular signatures of slow motoneurons over fast (Chakkalakal et al. 2010). Recent molecular profiling studies of adult motoneurons have also revealed additional molecular markers that distinguish between multiple motoneuron functional subtypes (Alkaslasi et al. 2021; Blum et al. 2021), opening up a means to resolve the developmental mechanisms through which these features are specified.

6 Evolution of Spinal Motoneuron Organization and Function

Animals exhibit a remarkable diversity in the types of motor behaviors they display. Evolutionary changes in motor behaviors allows animal to optimize the means through which they seek out mates, find food, and escape predators. Cross-species comparative analyses of the molecular and anatomical features of motoneuron subtypes can provide insights into how locomotor circuits may have evolved (Fetcho 1992; Jung and Dasen 2015; Murakami and Tanaka 2011). Even within a single species, genetic analyses of transcription factor function can provide insights into the origins of specific neuronal subtypes. For example, analyses of mice lacking *Foxp1* suggest that limb-innervating LMC neurons evolved from an HMC-like precursor that acquired sensitivity to Hox transcription factor activity (Dasen et al. 2008; Rousso et al. 2008).

Origins of Tetrapod Limb Motoneurons

The ability to travel on land was a key step in the evolution of vertebrates, and many of the circuit elements necessary for limb-based locomotion are contained within the spinal cord (Arber 2012; Goulding 2009; Grillner 2006; Kiehn 2016). It is thought that tetrapod locomotion evolved through a gradual transformation of a spinal network used to activate axial muscles during undulatory swimming, to a more localized circuitry dedicated to coordinating muscles in the limbs (Grillner and Jessell 2009; Jung and Dasen 2015). This idea is supported by the observation that contemporary representatives of intermediate steps in tetrapod evolution, including amphibians and reptiles, often display locomotor behaviors that are a composite of these two fundamentally distinct forms of locomotion (Chevallier et al. 2008).

However, in certain fish species forward propulsion is driven by oscillatory waves of muscle contraction within the fins, and in some cases, fins can be used to generate bipedal-like locomotor gaits (Holst and Bone 1993; Koester and Spirito 2003; Rosenberger 2001). In the little skate *Leucoraja erinacea,* the pelvic fins are used in locomotor behaviors displaying hallmark features of ambulatory locomotion, including reciprocal extension and flexion of the fins that alternate between the left and right sides of the spinal cord (Lucifora and Vassallo 2002; Macesic and Kajiura 2010). Skates generate this behavior using motoneuron subtypes that are remarkably similar to those found in tetrapods. Both pectoral and pelvic fin-innervating motoneurons of skates express Foxp1, and the Lim HD code essential for selective innervation of flexor and extensor muscles (Jung et al. 2018). Because the common ancestor of skates and tetrapods was the common ancestor to all vertebrates with paired appendages, these observations indicate that LMC neurons originated in ancient fin-bearing species (Fig. 5a).

Hox Genes and the Evolution of Motoneuron Segmental Organization and Diversity

Comparisons of the Hox-regulatory networks between vertebrates displaying different locomotor behaviors can provide mechanistic insights into how nervous systems may have evolved. Snakes evolved from limb-bearing reptiles that have repressed limb development and have reverted to an axial muscle-based form of locomotion (Leal and Cohn 2018). In African house and corn snakes, expression of the LMC determinants Foxp1 and Raldh2 are largely absent from the spinal cord, while axial MMC and HMC neurons are present throughout most spinal segments (Jung et al. 2014). This reversion of motoneurons to an almost entirely axial system is associated with a loss of brachial *Hox* gene expression from motoneurons and an expansion in the domain of *Hoxc9* expression within the spinal cord (Fig. 5b, c). As in other tetrapods, Hoxc9 likely acts in snakes by repressing anterior *Hox* genes and

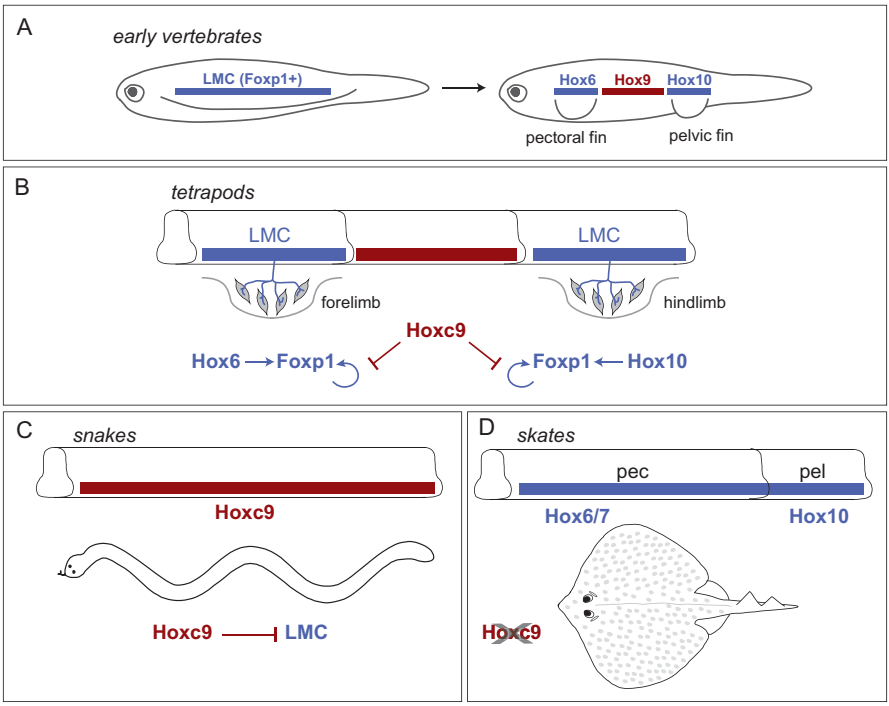

Fig. 5 Evolution of motoneuron diversity. (**a**) Model indicating that ancestral fin-bearing fish had LMC-like neurons, characterized by Foxp1 expression. This population may have extended along the length of the trunk. Hoxc9 activity was involved in generating separate pectoral and pelvic fin-innervating LMC populations, through repressing Foxp1 expression. (**b**) Regulatory interactions between Hox and Foxp1 in tetrapods. Limb level Hox proteins promote a high level of Foxp1 expression, which is maintained through Foxp1 autoregulation. Hoxc9 acts by blocking the ability of Foxp1 to positively autoregulate its own expression. (**c**) Evolutionary modifications in Hox-regulated organization of motor columns. In snakes, which have suppressed limb development programs, expression of Hoxc9 extends throughout most segments, and LMC neurons are not detected. (**d**) In skates, pectoral LMC neurons have expanded, likely as consequence of a natural deletion in the *HoxC* cluster, which removes the *Hoxc9* gene

Foxp1. Interestingly, this repressive activity is present in *Hoxc9* proteins of both terrestrial and aquatic vertebrate species (Jung et al. 2014), suggesting that Hox-dependent patterning is an ancestral strategy through which spinal motoneurons are organized.

In contrast to the loss of LMC neurons in snakes, in the little skate there is an extended domain of LMC neurons, spanning continuously from the pectoral fin-innervating population to the caudal pelvic domain. Skates also have a natural deletion in the *Hoxc9* gene (King et al. 2011), which likely contributed to the expanded domain of pectoral fin-innervating motoneurons (Fig. 5d). The pectoral and pelvic LMC neurons of skate also express Hox proteins that are analogous to those of tetrapods (Fig. 5d). These observations are consistent with a model in which the

ancestral LMC population was present throughout the spinal cord, with Hoxc9 activity being important for the generation of separate rostral (pectoral/forelimb) and caudal (pelvic/lumbar) LMC populations (Fig. 5a). Thus, modulation in the pattern of Hox expression is associated with evolutionary changes in motoneuron organization and locomotor behaviors.

The appearance of digits represents an important evolutionary modification of the limb, enabling fine motor skills and dexterity. The genetic programs that specify forelimb digit-innervating motoneurons involves a divergent Hox-dependent program that is deployed near the boundary between brachial LMC and thoracic subtypes. Digit motoneurons can be distinguished from other brachial motor pools by low levels of *Hoxc9* expression, as well as digit motor pool restricted markers (e.g. *Cpne4*, *Fign*) (Mendelsohn et al. 2017). Although high levels Hoxc9 normally represses LMC specification, at reduced levels it appears to only repress Raldh2, allowing for the generation of motoneurons that express Foxp1, and the caudal LMC determinant Hoxc8. Specification of forelimb digit-innervating motoneurons requires Hoxc8 and Hoxc9 and evasion of RA signaling, as elevation of RA signaling represses digit motoneuron specification. Analysis of digit motoneuron specification have revealed how changes in the levels and activities of Hox proteins can contribute to the evolutionary diversification of neuronal subtypes.

Divergence of Axial Motoneuron Specification Programs in Fish

In most species of fish, forward propulsion is governed by motoneurons that innervate axial muscle (Fetcho 1987; Romer and Parsons 1977). Although used for distinct motor behaviors, the axial motoneurons of fish and tetrapods share common early developmental programs, including reliance on Mnx1 and Lim HD proteins for the specification of motoneuron class identity. Despite sharing some common specification programs, zebrafish has diverged significantly from mammals, particularly in the programs specifying the subtype identities of axial muscle-innervating motoneurons.

In contrast to the motoneurons of tetrapods, axial motoneurons of zebrafish lack a clear somatotopic organization that relates cell body position to muscle target specificity. Despite the absence of an obvious somatotopic organization, zebrafish axial motoneurons are functionally organized along the dorsoventral axis. This organization is associated with how motoneurons are recruited at specific swimming speeds. Axial motoneurons active during slow swimming speed reside ventrally in the spinal cord, motoneurons recruited at fast swimming speeds are located dorsally, while motoneurons involved in intermediate speeds are positioned between fast and slow motoneurons (Ampatzis et al. 2013; Liu and Westerfield 1988; McLean et al. 2007).

Unlike mammals and birds, where all motoneurons are generated during a single wave of neurogenesis, zebrafish motoneurons are generated over two waves, termed primary and secondary. Studies of motoneuron specification in zebrafish have

largely focused on the four primary motoneuron types: the dorsal rostral primary (dRoP), ventral rostral primary (vRoP), caudal primary (CaP), and middle primary (MiP) neurons. dRoP and MiP motoneurons are similar to MMC neurons, in that they project to muscles located dorsal to the horizontal myoseptum, while CaP and vRoP project ventrally. However, unlike tetrapod MMC and HMC neurons, zebrafish primary motoneuron types cannot be distinguished by differential expression of Lhx3. Nevertheless, disruption of the core determinants Lhx3/4, Isl1/2, and the Mnx1 causes defects in primary motoneuron specification and connectivity. For example, loss of Lhx3/4 leads to motoneurons with hybrid motoneuron/interneuron fates (Seredick et al. 2014), while loss of Mnx proteins affects the specification of MiP motoneurons (Seredick et al. 2012).

Little is known about the specification of the later-born and more numerous secondary motoneurons (Myers et al. 1986). Secondary axial motoneurons are the most diverse, comprising at least 16 distinct subtypes, as classified by electrophysiological properties and morphological features (Bello-Rojas et al. 2019; Menelaou and McLean 2012). Although markers including Isl1, Isl2, and Mnx proteins can differentiate primary motoneuron subtypes, these factors are dynamically expressed and cannot distinguish secondary subtypes throughout development (Appel et al. 1995; Hutchinson et al. 2007; Seredick et al. 2012). Although secondary motoneurons make up the majority of subtypes in zebrafish, and are thought to be more similar to mammalian motoneurons, very little is known about the intrinsic TF codes responsible for their differentiation (Beattie et al. 1997).

Both primary and secondary motoneuron subtypes can be differentiated based on several criteria, such as birthdate, soma size, position, presence or absence of intraspinal or intermyotomal collaterals, and firing properties (Bello-Rojas et al. 2019; Menelaou and McLean 2012). There are three distinct types of firing patterns expressed by embryonic zebrafish axial motoneurons, tonic, chattering, and burst firing. Tonic firing patterns are specific to primary motoneurons, while chattering and burst firing patterns are specific to secondary motoneurons. Each secondary motoneuron subtype has a different distribution of these two firing patterns. While the distinct physiologic and anatomic features of secondary motoneurons have been well-characterized, it is yet unknown whether they reflect the operation of motoneuron-intrinsic genetic programs acting during development. Given that axial motoneurons are organized by functional attributes (fast, intermediate, and slow), as opposed to muscle-target specificity, and are present throughout the rostrocaudal axis, it is unlikely that their diversification relies on Hox-dependent differentiation programs. As with the differentiation of tetrapod MMC and HMC neurons, zebrafish motoneuron patterning may depend on signaling from the floorplate, or through cell-cell interactions between newly born neurons.

7 Assembly of Proprioceptive Sensory-Motor Circuits

Coordinate motor behavior depends on the establishment of central connections between motoneuron subtypes and premotor spinal and supraspinal circuits. While significant advances have been made in deciphering the mechanisms of motoneuron diversification and peripheral target specificity, the programs which determine the central connectivity of motoneurons are less well defined. Recent technological advancements in transsynaptic labeling methods, as well as the ability to selectively manipulate motoneuron subtype identities, have enabled investigation into the mechanisms of central synaptic specificity in spinal motor circuits. These studies provide evidence that motoneurons are not passive participants of motor commands, but can play instructive roles in shaping connectivity and activity of motor networks.

One of the most thoroughly studied circuits within the spinal cord is the monosynaptic stretch-reflex circuit, consisting of a limb muscle, a pool of alpha-motoneurons, and type Ia proprioceptive sensory neurons (pSNs) with stretch-sensing mechanoreceptor endings embedded within muscle spindles (Fig. 6a) (Imai and Yoshida 2018; Tuthill and Azim 2018). Despite its apparent simplicity, each motor pool receives selective inputs from pSNs that target the same peripheral muscle, suggesting a requirement for programs within pSNs to distinguish between different motoneuron subtypes. During development, pSN axons navigate through the spinal cord, preferentially contacting motoneuron pools innervating the same peripheral target, while avoiding motoneurons of functionally antagonist muscles (Eccles et al. 1957; Mears and Frank 1997). These connections are remarkably selective, as a single pSN establishes monosynaptic connections with each of the ~50–300 neurons within a motor pool that supplies the same muscle target (Mendell and Henneman 1968). Connections between pSNs and motoneurons appear to be established independent of patterned neural activity (Mendelsohn et al. 2015; Mendelson and Frank 1991), suggesting pSN-motoneuron matching relies on genetic programs operating during neural development. An important and still largely unanswered question is whether pSNs acquire molecular features that allow them to recognize specific motoneuron subtypes.

Fig. 6 (continued) and have central connections to alpha motoneurons that target the same muscle. A central connection between a flexor motoneuron and pSN is shown. Flexor pSNs synapse onto to flexor motoneurons, but not extensor motoneurons. (**b**) Consequences of *Foxp1* mutation on sensory-motor connections. In *Foxp1* mutants, motor pool organization is disrupted. Individual motoneurons can still target limb muscles their position is disorganized in *Foxp1* mutants. Nevertheless, pSN still project to the same dorsoventral domain in the ventral spinal cord. (**c**) Effect of *Hoxc9* mutation on sensory-motor connections. In *Hoxc9* mutants, ectopic LMC neurons are generated in thoracic segments and innervate hypaxial muscle. In *Hoxc9* mutants, limb-innervating pSNs project to, and form synapses on thoracic LMC neurons. (**d**) Coordinate *Hox* function in proprioceptive sensory-motoneuron connectivity. The same *Hox* genes expressed in motoneurons are selectively expressed by pSNs. Summary of Hoxa5 and Hoxc8 in brachial LMC neurons is shown in the top panels. After conditional deletion of *Hoxc8* from pSNs, flexor pSNs form connections with extensor motoneurons

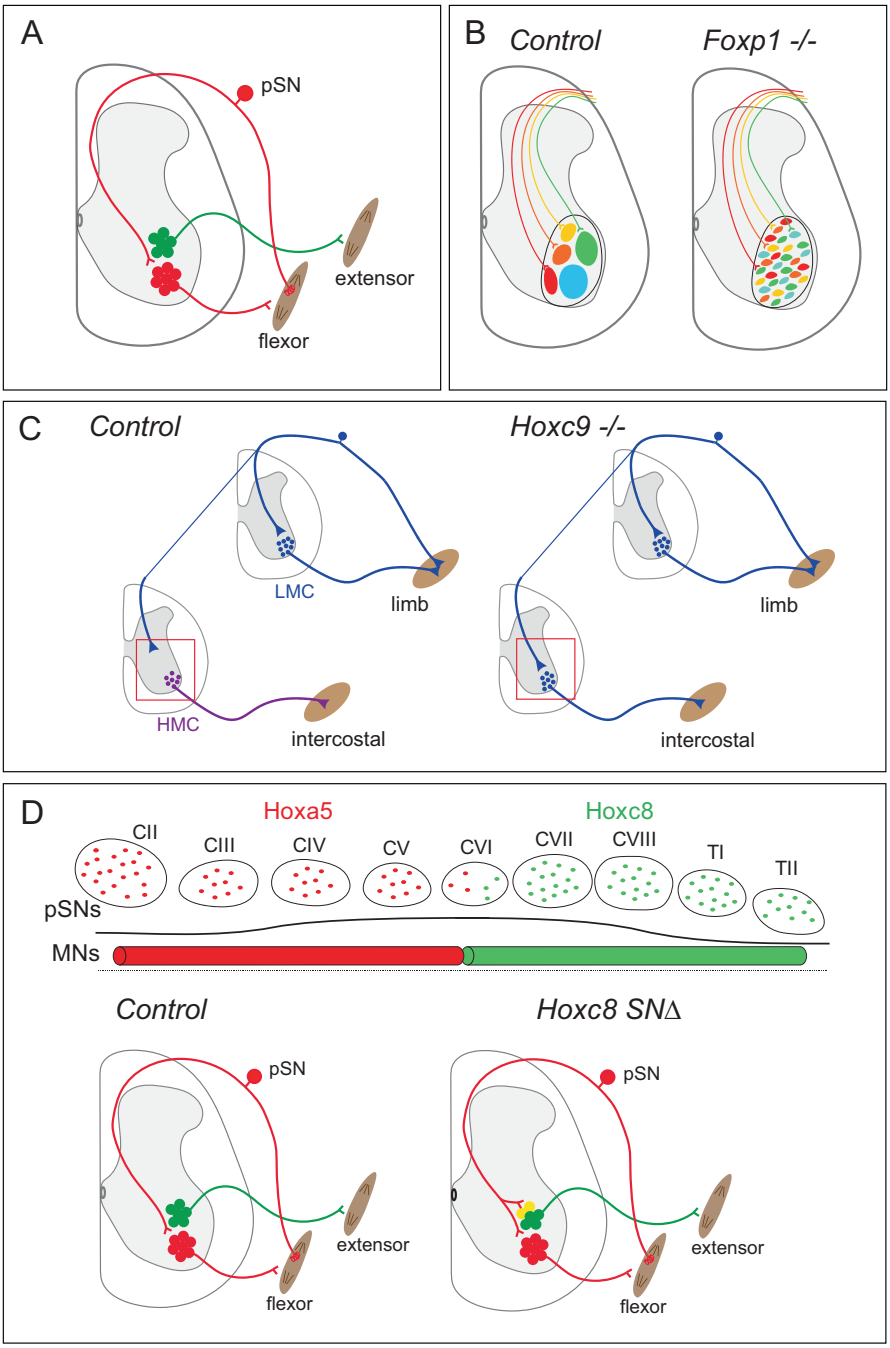

Fig. 6 Assembly of proprioceptive sensory-motor circuits. (**a**) Connections in the monosynaptic stretch reflex circuit. Type Ia proprioceptive sensory neurons (pSNs) innervate muscle spindles

Specification of Proprioceptor Sensory Neuron Class Identities

Similar to the diversification of spinal motoneurons, pSNs advance through hierarchical genetic programs in which expression of specific genes coincides with the acquisition of specialized characteristics, including peripheral target specificity, central projection pattern, and molecular identity (Dasen 2009; Lallemend and Ernfors 2012). Sensory neurons generated at spinal levels derive from migratory neural crest cells which coalesce to form dorsal root ganglia (DRG) (Butler and Bronner 2015). Most sensory neurons co-express the homeodomain transcription factors Isl1 and Brn3a, which are necessary for deployment of pan-sensory neuron genetic programs (Dykes et al. 2011).

Proprioceptive sensory neurons can be discriminated from other DRG sensory neuron classes by expression of the transcription factors Runx3 and Etv1, the neurotrophin receptor *Nrtk3*, and the calcium binding protein Parvalbumin (PV). Genetic studies in mice indicate that *Runx3* and *Etv1* are essential for establishing and maintaining core features of pSN identity, including their survival and ability to extend central axons towards motoneurons (Arber et al. 2000; Inoue et al. 2002).

While the transcriptional programs governing features common to all pSNs have been characterized, understanding later developmental facets of pSN-motoneuron circuit assembly, such as central connectivity with motoneurons, has been particularly challenging. In contrast to the topographic organization of motoneurons, pSNs are intermixed with other DRG sensory classes, with no clear organizational pattern (Honig et al. 1998; Jessell et al. 2011). A dearth of molecular markers for more nuanced neuronal features has made it challenging to characterize how pSNs and other sensory modalities further diversify into specific subtypes. One particular gap in our understanding is how the specificity of central connections between pSNs and motoneurons of the same muscle is achieved, since pSN axons must distinguish between multiple potential postsynaptic targets within the ventral spinal cord.

Target-Derived Signals Regulate pSN Subtype Identity and Connectivity with Motoneurons

In contrast to motoneuron specification, where early developmental features emerge largely independent of peripheral cues, pSN subtype specification relies on extrinsic signals provided by limb mesenchyme and muscle (Arber 2012; Sharma et al. 2020; Wu et al. 2019). Expression of the neurotrophin receptor *Nrtk3* renders pSNs sensitive to peripheral neurotrophin-3 (*Ntf3*) signaling, and both *Nrtk3* and *Ntf3* are essential for the differentiation and survival of pSNs (Chen et al. 2003). *Ntf3*/*Nrtk3* signaling regulates expression of *Etv1* and *Runx3*, and muscle-by-muscle

differences in the level of *Ntf3* expression appear to contribute to pSN subtype identities (de Nooij et al. 2013; Patel et al. 2003; Wang et al. 2019). In addition, signals originating from the limb mesenchyme can trigger expression of genes that mark muscle-specific pSN subtypes (Poliak et al. 2016). Although certain features common to all pSNs have been shown to be limb-independent (Chen et al. 2002), a predominant mechanism of pSN fate specification appears to be through muscle-specific cues.

Target-derived signals also regulate aspects of motoneuron pool identity, and can contribute to the specificity of connections between motoneurons and pSNs. Expression of the transcription factor *Pea3* is induced in brachial and lumbar LMC neurons by limb-derived neurotrophins. In the absence of *Pea3* function, pSNs target inappropriate motoneuron subtypes (Livet et al. 2002; Vrieseling and Arber 2006). Pea3 regulates expression of the guidance receptor ligand *Sema3e*, and in *Sema3e* mutants, pSNs target inappropriate motoneurons (Fukuhara et al. 2013; Pecho-Vrieseling et al. 2009). Although target-induced expression of guidance determinants is one strategy for controlling specificity in reflex circuits, they appear to operate in only a limited number of motoneuron pools.

Roles of Motoneuron Subtype Identity in Sensory-Motor Circuit Assembly

While studies of Pea3 function indicate that motoneuron identity can influence sensory -motor connectivity, some aspects appear to be motoneuron-independent. Mutation in the Hox-dependent transcription factor *Foxp1* strips LMC neurons of motor pool-specification programs, and while motoneurons are still able to innervate muscle, motor axons select targets in a stochastic manner (Dasen et al. 2008; Rousso et al. 2008). Nevertheless, pSNs project to the appropriate dorsoventral position within the ventral spinal cord and synapse with motoneurons (Surmeli et al. 2011). These observations suggest that pSNs can project to specific regions within the ventral spinal cord in the absence of motoneuron subtype-specific cues (Fig. 6b). An interpretation of these findings is that one function of motoneuron topographic organization is to ensure the alignment of sensory central afferents with the corresponding motoneuron pools. Additional studies on the connections established between pSNs and spinal interneurons reinforce the idea that target cell position is involved in establishing synaptic specificity in spinal circuits (Bikoff et al. 2016; Tripodi et al. 2011).

Studies of *Foxp1* mutants suggest that motoneuron-derived cues do not play an instructive role in determining the dorsoventral targeting of pSN within the ventral spinal cord. However, because motoneurons are stripped of all LMC-specific features, it leaves open the possibility that pSNs are capable recognizing molecular differences among LMC neurons. In particular, the targeting of a pSN to its appropriate motor pool presumably also relies on the recognition of differences in

motoneurons along the rostrocaudal axis. One way to test this would be to extend the rostrocaudal domain of motoneuron pools, and assess the impact on pSN-motoneuron connectivity. Mutation in the *Hoxc9* gene transforms thoracic motoneurons to a limb-level LMC fate, and extends the domain of Hoxc8^{+} motor pools into thoracic segments. In *Hoxc9* mutants, transformed thoracic motoneurons receive input from forelimb-innervating pSNs (Fig. 6c) (Baek et al. 2017). Thus, while the dorsoventral targeting of pSNs in the spinal cord may be motoneuron independent, the selection of termination zones along the rostrocaudal axis appears to depend on recognition of Hox-mediated differences in motoneuron subtype identity.

Evidence for Coordinate Regulation of pSN-Motoneuron Connectivity by Hox Genes

While studies suggest that Hox-dependent programs are essential in motoneurons for aspects of sensory-motor specificity, whether pSNs acquire muscle-specific molecular features similar to those of motoneurons is not fully known. *Hox* expression in pSNs also displays segmentally-restricted patterns, paralleling the rostrocaudal profiles of *Hox* genes in motoneurons (Shin et al. 2020). Proprioceptive sensory neurons may therefore use similar diversification programs as motoneurons. For example, the *Hoxc8* gene is expressed by LMC neurons targeting distal forelimb muscle, and is also expressed by pSNs targeting similar muscle groups (Fig. 6d). Expression of *Hox* genes in sensory neurons also does not appear to require limb-derived cues, as limb ablation experiments in chick do not affect Hox expression in pSNs (Shin et al. 2020). Hox activity in pSNs may therefore operate to provide a limb-independent mechanism of neuronal diversification.

Genetic studies in mice reveal that Hoxc8 activity in pSNs contributes to sensory-motor synaptic specificity. Deletion of *Hoxc8* selectively from SNs does not affect neuronal survival or prohibit the ability of pSNs to target the correct forelimb muscle. However, in *Hoxc8* sensory mutants, flexor pSNs make inappropriate connections with extensor motoneurons (Shin et al. 2020). Similar defects in sensory-motor matching is observed when *Hoxc8* is deleted from motoneurons. These observations suggest that the coordinate activity of *Hox* genes in pSNs and motoneurons, acting in conjunction with muscle-derived cues, contributes to synaptic specify in sensory-motor circuits. The specificity of pSN-motoneuron connections has been shown to correlate with the relative approach angles between pSN central axons and motoneuron dendrites (Balaskas et al. 2019). Sensory axons approach the dendrites of motoneurons targeting the same muscle at small angles, relative to the approach angle to dendrites of other motoneuron subtypes, suggesting the angle of axo-dendritic approach contributes to connection specificity. Loss of pSN-motoneuron alignment may account for the sensory-motor mismatch observed in both motoneuron and pSN-restricted *Hoxc8* mutants.

Centrally, pSNs establish connections with a variety of postsynaptic targets, including local spinal and projection interneurons that relay proprioceptive information to the brain (Bermingham et al. 2001; Bikoff et al. 2016; Koch et al. 2017; Tripodi et al. 2011; Yuengert et al. 2015). The same *Hox* genes expressed by pSNs and motoneurons are also expressed by multiple classes of spinal interneurons, suggesting Hox proteins play a broader role in shaping synaptic specificity within the spinal cord. Consistent with this idea, both long ascending spinocerebellar and local-circuit spinal interneurons require *Hox* function to generate molecularly distinct subtypes at cervical and thoracic levels (Baek et al. 2019; Sweeney et al. 2018). These observations suggest that the same *Hox* gene can act in multiple neuronal classes during development, implying a coherent molecular strategy for wiring the circuits essential for motor control.

8 Motoneurons as Regulators of Circuit Assembly and Function

Dedicated circuits contained within the vertebrate brainstem and spinal cord are capable of generating rhythmic patterns of motoneuron activation essential for basic motor functions such as walking and breathing. In the circuits controlling locomotion, the rhythm and pattern of motoneuron activity is facilitated by central pattern generators (CPGs) composed of multiple classes of excitatory and inhibitory spinal interneurons (Grillner 2006; Grillner and Jessell 2009). In tetrapods, spinal locomotor CPGs coordinate alternating patterns of motoneuron firing across the left and right side of the spinal cord (L-R CPG) as well as reciprocal activation extensor and flexor motoneurons (E-F CPG). The spinal interneurons required for the pattern of CPG output in fictive locomotor preparations are well-defined (Goulding 2009; Kiehn 2016). Left-right alternation relies on V0-class interneurons which cross the midline and inhibit the contralateral CPG (Lanuza et al. 2004; Talpalar et al. 2013). Coordination of extensor-flexor motoneuron activity requires V1 and V2b class inhibitory interneurons (Britz et al. 2015; Zhang et al. 2014). Several additional classes of genetically defined spinal interneurons are essential to determine the robustness of CPG output as well as modulating motoneuron firing at different locomotor speeds (Kiehn 2016).

While the identity and function of the spinal interneurons involved in locomotor CPGs have been elucidated, how these premotor populations engage specific motoneuron subtypes is less well understood. Because a single motoneuron can receive inputs from a variety of presynaptic neuronal classes, and there are dozens of distinct motoneuron subtypes, it has been challenging to determine whether there are rules that govern connectivity between spinal motoneuron subtypes and locomotor CPG interneurons. One possible solution could be through generating interneurons of a similar diversity to that of motoneurons. Evidence in support of this idea has come from recent studies on the diversification of spinal interneuron subtypes,

revealing a remarkable degree of molecular heterogeneity within a single interneuron class (Bikoff et al. 2016; Griener et al. 2015; Hayashi et al. 2018).

Evidence suggesting a role for motoneuron subtype identity in the assembly of locomotor circuits has emerged from studies examining the distribution of interneurons that directly synapse onto motoneurons. The development of transynaptic tracing methods has enabled the mapping the distribution of inputs onto motoneurons (Esposito et al. 2014; Stepien et al. 2010). Analyses of the patterns of premotor connectivity have revealed marked differences in the types and distribution of interneurons that motoneuron subtypes engage. LMC neurons receive inputs that are largely biased towards spinal inhibitory interneurons localized ipsilaterally (Fig. 7a) (Goetz et al. 2015). In contrast, motoneurons projecting to axial muscles, including those located in the MMC and HMC, receive inputs that are evenly distributed across both sides the spinal cord, with a bias towards contralateral populations. Although interpretation of these findings is constrained by the inherent inefficiency of transsynaptic labeling methods, these observations suggest there are differences in the distribution of premotor interneurons that specific motoneuron columnar subtypes are connected with.

Differences in the distribution of premotor interneurons are also for observed for inputs onto motor pools that occupy specific regions within the LMC. Motoneurons situated ventromedially receive a greater proportion of inputs from contralateral premotor interneurons than motoneurons projecting to distal limb muscles, which are dorsolaterally positioned (Goetz et al. 2015). A suggested possible mechanism for these biases is through the configuration of motoneuron dendrites. MMC neurons, which are positioned medially, have dendrites which extend across the midline and can captures a greater proportion of input originating from contralateral interneuron populations. Recent studies also suggest a bias in the types of local interneurons that synapse onto motoneurons projecting to extensor and flexor muscles, which could similarly reflect differences in motoneuron position and/or dendritic morphology (Britz et al. 2015). Given that motor pool-restricted factors such as *Pea3* are known influence the pattern of motoneuron dendritic morphology (Vrieseling and Arber 2006), inputs from premotor interneurons could be shaped by both the positional and architectural features of motoneurons.

Motoneuron subtype identity has also been shown to influence activity of locomotor CPGs. The role of motoneurons subtype identity has been examined using mouse mutant lines in which motoneuron identities have been genetically transformed (Hinckley et al. 2015; Machado et al. 2015). Removal of *Foxp1* from motoneurons leads to an LMC to HMC transformation, but these motoneurons still target limb muscles. In motoneuron-specific *Foxp1* mutants, basic features of the CPG are retained, including normal rhythmic bursting patterns and left-right alternation. By contrast the output pattern from the E-F CPG is disrupted, reverting to a predominantly flexor-like state (Hinckley et al. 2015; Machado et al. 2015). In *Foxp1* mutants, transformed LMC populations are also shifted to a more ventromedial position. This raised the possibility that the defects in *Foxp1* mutants are due to changes in premotor connectivity as a consequence of altered motoneuron position. This idea was tested by misexpressing Lhx3 neurons in all motoneurons, a

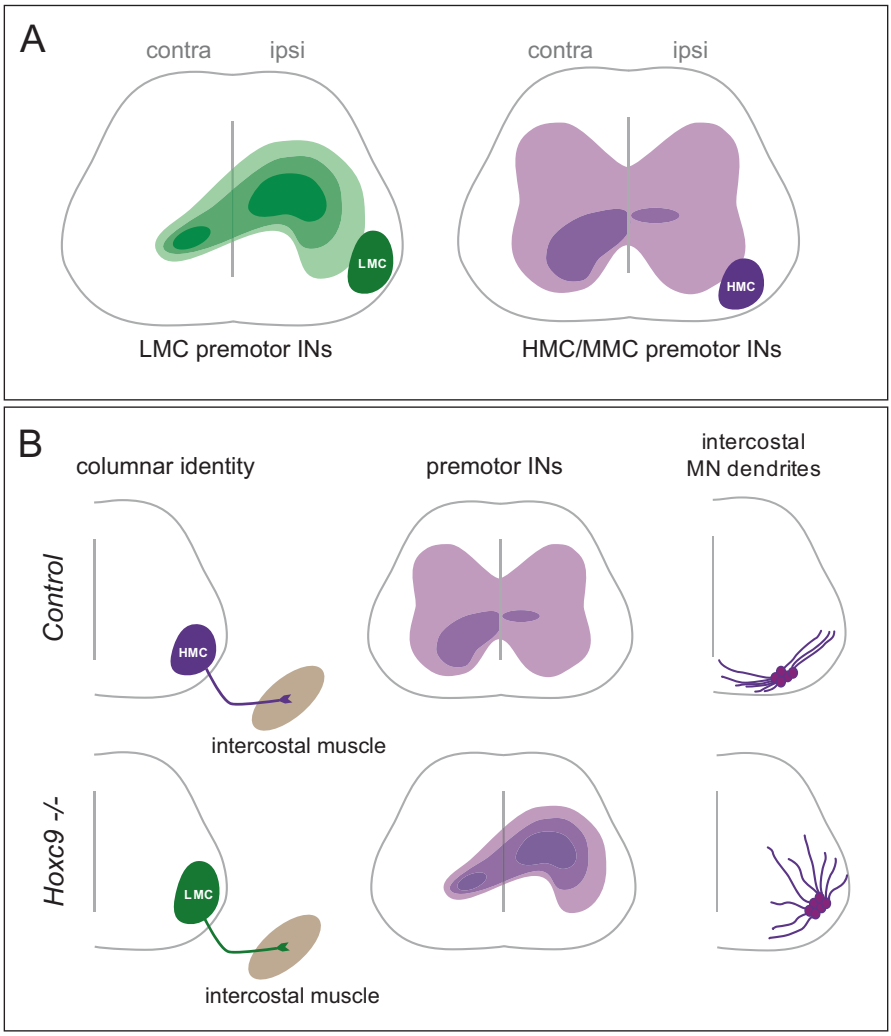

Fig. 7 Motoneurons in the development of locomotor circuits. (**a**) Pattern of premotor inputs onto LMC and MMC/HMC subtypes. LMC premotor interneurons are localized predominantly on the ipsilateral side of the spinal cord, whereas interneurons synapsing onto MMC and HMC neurons are equally distributed on both sides of the cord. Darker color shading indicates higher density of premotor inputs. (**b**) Effect of *Hoxc9* mutation on the distribution of premotor inputs. In *Hoxc9* mutants, thoracic HMC neurons are converted to an LMC fate. Ectopic LMC neurons project to intercostal muscle in *Hoxc9* mutants and dendritic morphology of thoracic motoneurons is altered. Thoracic motoneurons receive a higher distribution of inputs from ipsilateral premotor interneurons in *Hoxc9* mutants

manipulation that suppresses LMC differentiation and promotes an MMC identity (Dasen et al. 2008; Sharma et al. 2000). Many of these ectopic MMC neurons occupy the same position as LMC neurons, but still display a predominantly

flexor-like motoneuron bursting pattern (Hinckley et al. 2015). These observations suggest that position alone is not sufficient to determine the E-F pattern of motoneurons firing.

In addition to determining the pattern of output from E-F CPGs, motoneurons have also been demonstrated to retrogradely influence the firing properties of excitatory premotor interneurons. In zebrafish, motoneurons involved in slow, intermediate, a fast swimming have been shown to be connected to interneurons via gap junctions (Song et al. 2016). A major excitatory input to motoneurons derives from V2a interneurons that form monosynaptic connections with motoneurons, and V2a interneurons are key components of the rhythm-generating networks that drive zebrafish locomotion (Ampatzis et al. 2014; El Manira 2014). Interestingly, gap junctions occur not only between V2a and motoneurons, but also between groups of V2a neurons and groups of motoneurons. Hyperpolarization of motoneurons increases the firing threshold and decreases the firing frequency of V2a interneurons. Conversely, depolarization of motoneurons decreases V2a thresholds and increases their firing frequency. Backward propagation of electrical signals from motoneurons can a have important influence on the activities of CPG circuits, by setting the firing threshold and properties of excitatory V2a interneurons. In mammals, motoneurons have also been demonstrated to provide feedback to regulate the CPG rhythm in fictive locomotor assays, but not through gap junctions between motoneurons and interneurons (Falgairolle et al. 2017).

While studies show that motoneurons play an instructive role in defining functional properties of locomotor networks, the role of motoneuron identity in spinal circuit assembly has remained unclear. Similar to their roles in shaping the specificity between pSNs and motoneurons, Hox-dependent programs also can regulate the patterns of connectivity between spinal interneurons and motoneuron subtypes. After global or motoneuron-restricted removal of *Hoxc9*, which transforms thoracic HMC and PGC neurons to an LMC fate, the pattern of premotor inputs to these fate-transformed thoracic motoneurons is markedly altered (Baek et al. 2017). The distribution of intercostal premotor interneurons shifts to an ipsilateral bias, similar to patterns normally observed for limb-innervating LMC motoneurons (Goetz et al. 2015). These changes do not appear to be due to peripheral signals provided by the limb, indicating motoneuron-intrinsic programs regulate connectivity between spinal interneurons and columnar subtypes. Mutation in *Hoxc9* also alters the settling position and dendritic architecture of transformed motoneuron populations (Fig. 7b). The changes in interneuron premotor distributions in *Hoxc9* mutants therefore could reflect changes in both the molecular profile and somatodendritic features of motoneurons. These results are consistent with the view that aspects motor circuit assembly relies on both positional and molecular recognition cues (Jessell et al. 2011).

9 Conclusions

Studies of spinal motoneuron development have provided fundamental insights into the molecular programs underlying the diversification and synaptic specificity of a single neuronal class. Work on motoneurons has enabled exploration into basic questions in developmental neurobiology, such as how multipotent cells interpret morphogen gradients, how gene regulatory networks are deployed during differentiation, and how subtype specific gene programs determine neuronal synaptic specificity. These studies have revealed molecular mechanism that are applicable to other classes of neurons in the CNS. Further cross-species analysis, taking advantage of the relative simplicity of the neuromuscular system of skates, or the divergent functional organization of motoneurons in zebrafish, should yield novel insights into the mechanisms of motoneuron differentiation, and how motoneuron identities shape circuit assembly and function.

While modern molecular approaches have enabled unprecedented resolution in the characterization of subtype-specific gene expression patterns, there remain significant gaps in our understanding of how molecular features of motoneurons contribute to their connections and functional properties. One challenge is that motoneurons receive synaptic input from a daunting array of neuronal classes, including excitatory and inhibitory spinal interneurons, proprioceptive sensory neurons, neuromodulatory systems, and descending cortical inputs. While the mechanisms of premotor synaptic specificity are still not fully resolved, the ability to genetically manipulate motoneuron differentiation programs provides an entry point to further resolve how motor circuits are assembled during development. Recent findings reveal that the same *Hox* gene expressed by motoneurons are also required for the diversification of interneurons and proprioceptive sensory neurons, suggest that the use of a common group of regulatory factors may be an important mechanism regulating synaptic specificity in spinal circuits.

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