

Generating spinal motor neuron diversity: a long quest for neuronal identity

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Abstract Understanding how thousands of different neuronal types are generated in the CNS constitutes a major challenge for developmental neurobiologists and is a prerequisite before considering cell or gene therapies of nervous lesions or pathologies. During embryonic development, spinal motor neurons (MNs) segregate into distinct subpopulations that display specific characteristics and properties including molecular identity, migration pattern, allocation to specific motor columns, and innervation of defined target. Because of the facility to correlate these different characteristics, the diversification of spinal MNs has become the model of choice for studying the molecular and cellular mechanisms underlying the generation of multiple neuronal populations in the developing CNS. Therefore, how spinal motor neuron subpopulations are produced during development has been extensively studied during the last two decades. In this review article, we will provide a comprehensive overview of the genetic and molecular mechanisms that contribute to the diversification of spinal MNs.

Keywords Motor neurons · Spinal cord · Neuronal diversification · Cell differentiation · Embryonic development · Central nervous system

Abbreviations

Foxp	Forkhead box p
HMC	Hypaxial motor column
HNF-6	Hepatocyte nuclear factor-6
iPS	Induced pluripotent stem

LMC	Lateral motor column
MMC	Medial motor column
MN	Motor neuron
Neurog2	Neurogenin 2
OC	Onecut
PGC	Preganglionic column
pMN	Motor neuron progenitor

Introduction

“The neurons that constitute the motor cranial nerves and the spinal motor neurons (MNs) are the most important cells of the body” (*anonymous*). Although this provocative statement may at first glance sound severely overstated, a deeper analysis may eventually lead to reconsider this initial impression. Indeed, one may reasonably estimate that MNs constitute the single output of the CNS. Accordingly, whatever you think, dream, imagine, or want, and however smart or creative you are or particularly well connected your neuronal circuitries are, as long as you do not produce any action or any body movement, one may consider that the activity of your CNS will neither support your own survival nor impact on your environment and is therefore totally useless. Hence, MNs constitute a cell population of critical importance for animal and human life.

How these cells of paramount importance are generated during development has been extensively studied during the last two decades. In particular, the differentiation of spinal MN has become the model of choice for studying the mechanisms that promote the generation of different neuronal populations throughout the CNS. Indeed, a single well-defined progenitor domain generates in a restricted amount of time a collection of a few dozen MN subsets, each characterized by specific molecular

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markers, a defined location within a specific MN column along the anteroposterior axis of the developing spinal cord and, most importantly, a connectivity pattern with specific target muscles that has been exquisitely, although incompletely, deciphered. Recent articles have provided excellent overviews of MN diversity, survival, positioning, axon pathfinding, and connectivity [1–7]. The goal of the present review is to comprehensively summarize past and recent advances in the understanding of genetic and molecular mechanisms that contribute to the diversification of spinal MNs.

Spinal MN diversity

Motor neurons are efferent neurons, i.e., characterized by axonal projection leaving the CNS to reach specific targets in the periphery. All the vertebrate spinal MNs share many common features including large cell body size, neurotransmitter phenotypes, exit route from the CNS through ventral exit points, etc. However, they differentiate early in development into diverse subsets with specific characteristics. Multiple criteria can be used to underline spinal MN diversity. The broadest division segregates MNs that innervate the skeletal muscles of the body, namely somatic MNs, from those that regulate the contraction of the smooth muscles of the visceral organs, called visceral MNs. Neurons from each of these divisions gather at different locations within the spinal cord (Fig. 1). Indeed, visceral MNs are found exclusively at thoracic levels of the spinal cord in a medio-lateral column called the pre-ganglionic column (PGC). The neurons of this column innervate the sympathetic ventral chain wherein they make synapses with the noradrenergic neurons of the paravertebral ganglia, hence its name, and regulate cardiac or smooth muscle contractions and exocrine or endocrine gland activity. In contrast, somatic MNs locate in different columns showing variable extent along the anteroposterior axis of the spinal cord according to the set of skeletal muscle they innervate. MNs that control the axial muscles of the back are found in the medial motor column (MMC), which extends along the entire spinal cord. Neurons that innervate the body wall muscles are located in the hypaxial motor column (HMC), which is present at thoracic levels. In addition, evidence for the presence of a small complement of neurons that display a HMC phenotype at brachial and at lumbar levels has been provided [8, 9], although the target muscles innervated by these MNs remain unknown. Finally, MNs that regulate the activity of the locomotive muscles, including limb muscles and some back muscles that contribute to the movements of the pectoral and of the pelvic girdle, are restricted to the lateral motor columns (LMC), which are exclusively found at the levels of the limbs in the brachial or lumbar

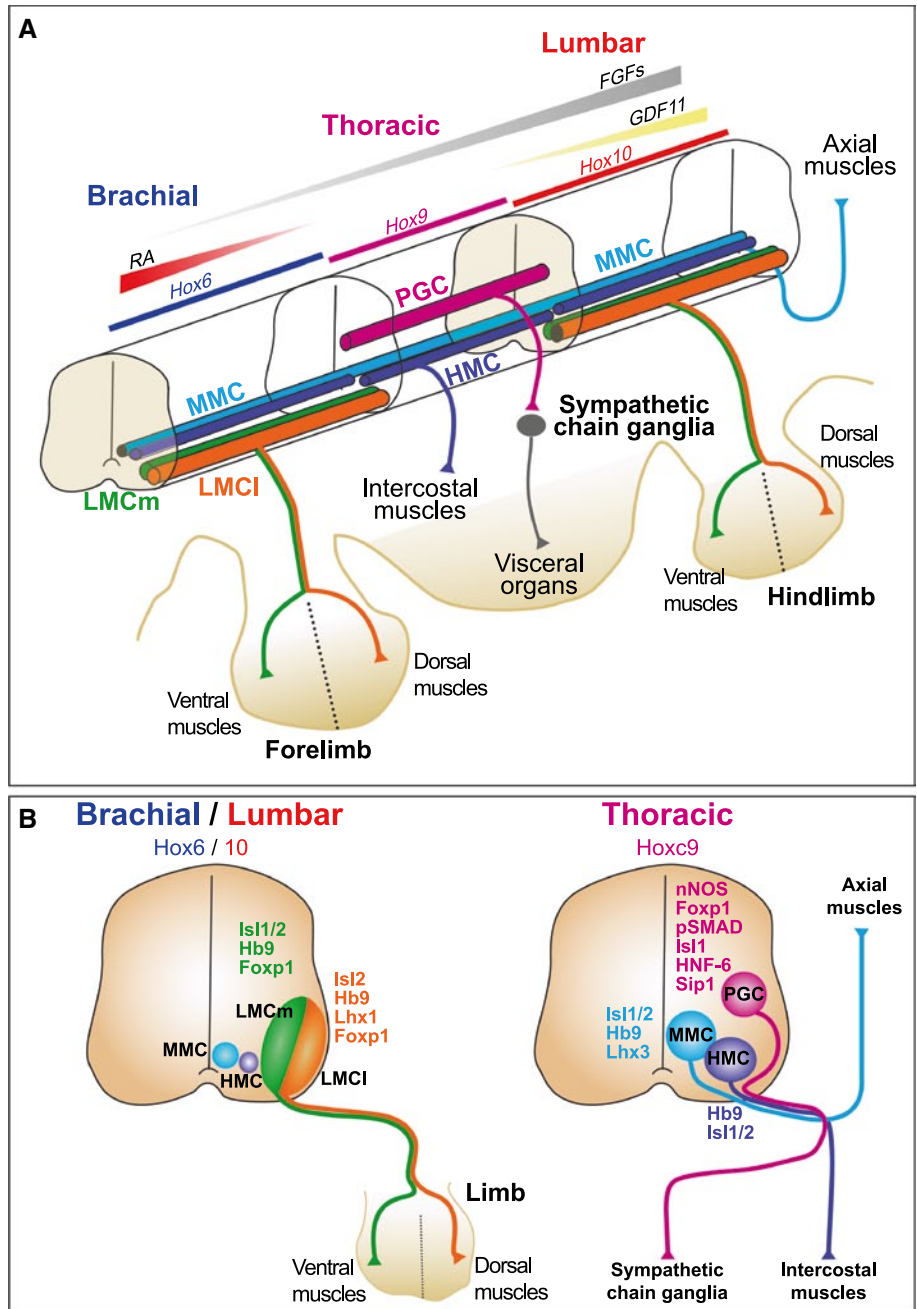
regions of the spinal cord. Among these, the medial complement of the LMC neurons (LMCm) innervates the muscles generated in the ventral portion of the developing limb buds, mainly flexor muscles, while the lateral complement (LMCl) connects to muscles generated in the dorsal portion of the limb buds, mainly extensor muscles (Fig. 1).

Additional levels of diversity include the existence of alpha, beta, and gamma MNs based on the type of muscle fibers (fusar, extrafusar, or both) they innervate, and of different subtypes of alpha MNs that can be classified according to the contractile properties of the motor units that they form with target muscle fibers (fast-twitch fatigable, fast-twitch fatigue-resistant, or slow-twitch fatigue-resistant) (reviewed in more detail in [2]). Finally, it has been demonstrated in the brachial and in the lumbar LMC that MNs that innervate the same muscle gather in so-called motor pools and share identical molecular properties including transcription factor expression profile [10–13], axon guidance and adhesion molecules [14], and neurotransmitter receptors [15]. Whether similar motor pools also exist at thoracic levels and innervate single axial or body wall muscles remains to be determined. Hence, MN is a generic term that actually designates many different neuronal types, each able to modulate the contraction of its specific target muscles in a specific manner. This, as well as the tremendous advances in the understanding of the genetic and molecular mechanisms that drive the diversity of this particular neuronal population, made MNs the model of choice for studying neuronal diversification in the developing CNS. Indeed, all the spinal MNs derive from a single progenitor domain (pMN) located in the ventral portion of the ventricular zone along the anteroposterior axis of the spinal cord. In the next sections, we will briefly summarize the mechanisms that drive the generation of newly born MNs before elaborating on the factors that promote the diversification of this neuronal population during embryonic development.

From pMN progenitors to newly born MNs

Extrinsic cues initiate the spatial patterning of the neuroepithelium in the developing spinal cord. Along the ventro-dorsal axis, Sonic Hedgehog (Shh) initially secreted by the notochord and subsequently produced by the cells of the floor plate provide a positional information that is required for the generation of ventral neuronal populations and triggers the subdivision of the neural progenitors into distinct ordered domains [16, 17]. Increasing concentrations of Shh and progressively longer exposures to Shh signaling impose gradual specification as more ventral progenitor populations [18, 19]. Indeed, high and prolonged Shh levels promote the expression of class II homeobox genes coding for *Nkx2.2*, *Nkx6.1*, *Nkx6.2*, or *Pax6* in ventral

Fig. 1 Topographic organization and molecular markers of motor columns along the anteroposterior axis of the developing spinal cord. **a** Schematic diagram illustrating the three-dimensional distribution of the motor columns. Graded levels of the morphogens FGFs, RA, and GDF11 determine spatial expression of *Hox* paralog groups that shape the spinal cord into a brachial, a thoracic, and a lumbar portion. At thoracic levels, MNs are distributed in a medial motor column (MMC) that innervates the axial muscles, a hypaxial motor column (HMC) connected to the intercostal muscles and a column of preganglionic MNs (PGC) that project to the sympathetic ganglia chain, which innervates visceral organs. At brachial and lumbar levels, MNs gather in an MMC column, a restricted HMC column, and a lateral motor column (LMC) that innervates locomotor muscles. MNs of the LMC are divided into a medial (LMCm) and a lateral (LMCl) complement that innervate ventral or dorsal limb muscles, respectively. **b** Location of each motor column and their molecular markers on schematic transverse sections in embryonic spinal cord at brachial/lumbar or at thoracic levels. *Hox6* are determinants of the brachial MNs and *Hox10* are required for lumbar MN fate, while *Hox9* establishes thoracic MN identity. *FGFs* fibroblast growth factors, *RA* retinoic acid, *GDF11* growth differentiation factor 11



regions of the neural tube while low levels and brief activation of the Shh pathway is permissive for the expression of class I homeobox genes including those coding for *Dbx1*, *Dbx2*, or *Irx3*, which are therefore restricted to more dorsal regions [18–20]. Molecular interplay between these transcriptional repressors ensures the restriction of *Nkx2.2* to the ventral-most progenitor cells that will give rise to V3 interneurons, and of *Irx3* to more caudal domains that will produce other ventral interneurons, resulting in the creation of a domain expressing *Pax6* and *Nkx6* genes that is permissive for MN differentiation [21, 22]. In this domain,

retinoic acid (RA) triggers the expression of *Olig2*, the hallmark of MN and oligodendrocyte progenitors. Indeed, spinal oligodendrocytes are generated from the same progenitor domain at later developmental stages [23–25].

Retinoic acid produced by the paraxial mesoderm is required, independently from Shh signaling [26], for neurogenesis in the developing spinal cord [27–29]. Indeed, RA contributes to high expression levels of homeobox genes, including *Irx3* and *Pax6*, and thereby enhances the patterning activity of Shh and contributes to define the pMN domain [28]. In more dorsal regions, it also promotes the

generation of V0 and V1 interneuron progenitors and the production of differentiated V1 neurons [26]. Subsequently, RA activates its cognate receptors in the pMN and initiates the expression of *Olig2* [28]. Although initially coexpressed with *Nkx2.2* or *Irx3* in cells that will eventually locate in the adjacent progenitor domains, the expression of *Olig2* is rapidly refined through mutual cross-repression with *Nkx2.2* or *Irx3* to become strictly restricted to the MN progenitor domain [25, 30]. Additional factors, including Wnt signaling, which interacts with the Shh pathway to modulate *Nkx2.2* expression [31–33], and the Groucho/transducin-like Enhancer of split proteins [34] also contribute to define the ventral boundary of the pMN domain. In turn, recent evidence demonstrates that the interpretation of the Shh gradient, which is continuously at play during this whole process, relies on a gene regulatory network that involves *Pax6*, *Olig2*, and *Nkx2.2* [35].

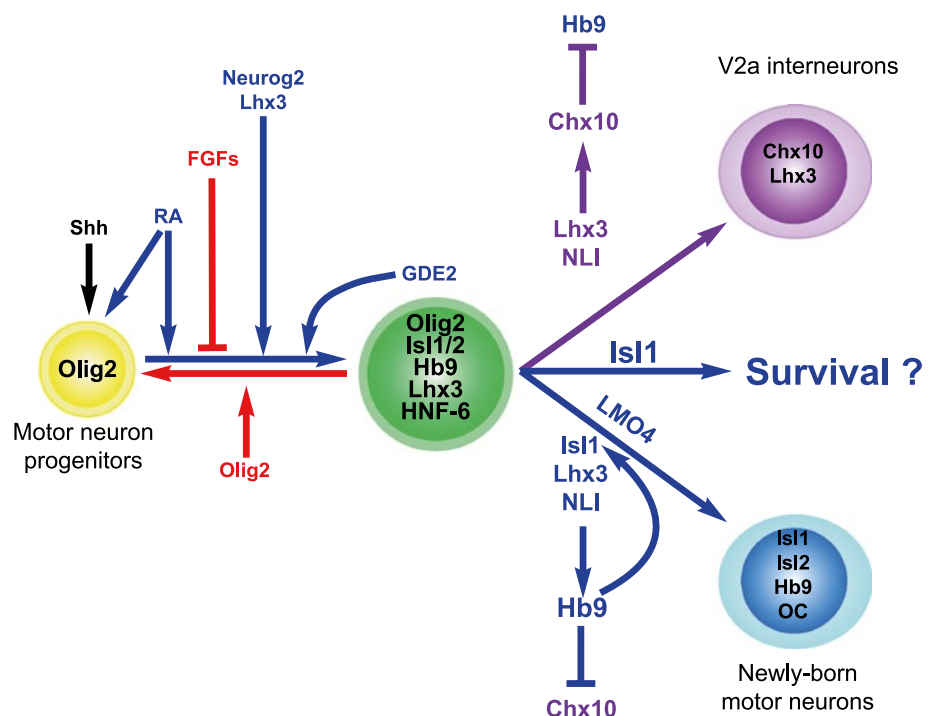
In the pMN domain, *Olig2* combined to Shh signaling rapidly stimulates the expression of the pro-neural gene *Neurogenin2* (*Neurog2*) (Fig. 2) [21, 25, 36]. This increase in *Neurog2* protein levels will determine the onset of MN differentiation. Indeed, the *Olig2/Neurog2* ratio serves as a gate for timing proper gene expression during the development of MN progenitors. High levels of *Olig2* maintain these cells in a progenitor state, whereas high levels of *Neurog2* favor their conversion into post-mitotic MN [21, 37, 38]. Except for the MN determinant *Hb9* (see below), the expression of which is directly repressed by *Olig2* [37], the physiological targets of *Olig2* and *Neurog2* in MN progenitors remain unknown. However, the LIM-HD

proteins *Lhx3* might be downstream of *Olig2* [21]. Consistently, *Lhx3* and its homolog *Lhx4* are jointly required for the generation of spinal MNs (Fig. 2) [39]. The transition from MN progenitors to differentiating neurons is further promoted by the activation of *Foxp2* and *Foxp4* that cooperatively repress the expression of N-cadherin and thereby promotes the detachment of differentiating MN from the neuroepithelium [40].

Epigenetic mechanisms also contribute to the onset of MN differentiation. Indeed, *Neurog2* forms with non-activated RA receptors a complex that is recruited on E boxes of MN genes targeted by *Neurog2*. Upon stimulation by RA, this complex promotes neurogenesis via the recruitment of *CBP*, a transcription factor with histone acetyltransferase activity, which in turn induces chromatin alterations in MN genes and activation of their transcription. Accordingly, tissue-specific inactivation of *CBP*, either alone or in combination with that of its paralog gene *p300*, results in alterations in MN generation [41]. Noticeably, these mutant embryos also showed perturbations in MN migration and axonal projections, suggesting that epigenetic modifications also regulate later aspects of MN development. It should be noted that *CBP* and *p300* are, in liver cells, cofactors of other transcriptional regulators that participate in MN diversification (see below), including One-cut factors [42].

Besides these cell-autonomous processes initially triggered by extrinsic cues, recent observations suggest that non-cell autonomous interactions between MN progenitors and post-mitotic neurons also participate in MN generation.

Fig. 2 MN fate specification and consolidation. A combination of extrinsic signals including Shh, RA, and FGFs cooperate with intrinsic mechanisms that involve *Olig2*, *Neurogenin-2* (*Neurog2*), and *Lhx3* to promote the specification of newly born MN. *GDE2* produced by the newly born MN acts in an on-cell autonomous manner to promote MN progenitor differentiation. *Lhx3*, *Isl1*, *NLI*, *LMO4*, and *Hb9* act together to consolidate MN identity. *Isl1* may additionally contribute to the survival of newly born MNs. *FGFs* fibroblast growth factors, *RA* retinoic acid, *Shh* Sonic Hedgehog, *NLI* nuclear Lim-domain interactor, *GDE2* glycerophosphodiester phosphodiesterase 2



Indeed, in parallel to multiple roles in spinal progenitors (see above), RA stimulates in post-mitotic MNs the expression of the glycerophosphodiester phosphodiesterase GDE2, which turned out to be necessary and sufficient for MN production [43–45]. GDE2 utilizes its extracellular glycerophosphodiester phosphodiesterase activity to induce MN generation by inhibiting, in a non-cell autonomous manner, Notch signaling in neighboring MN progenitors (Fig. 2) [45]. GDE2 activity requires the reduction by the antioxidant scavenger peroxiredoxin1 of an intramolecular disulfide bond that bridges its intracellular N- and C-terminal domains [46] and the formation of a complex with Gai-GDP, which acts in this context independently from its roles in transducing GPCR signals [44]. Thus, complex genetic and epigenetic processes initiated by extrinsic signals and that eventually give rise to non-cell autonomous crosstalks between differentiating neurons and progenitor cells direct the generation of MNs from the pMN domain.

Anteroposterior patterning of MNs by Hox factors and cofactors

The earlier step of MN diversification involves the activation of different genetic programs along the anteroposterior of the neural tube that eventually results in the formation of distinct motor columns at brachial, thoracic, or lumbar levels of the spinal cord. Indeed, the positional identity of neurons along the anteroposterior axis is established before the first differentiating neurons can be identified in the spinal cord [47], indicating that these characteristics are acquired and maintained by MN progenitors and transmitted to post-mitotic cells. Similar to the ventro-dorsal axis, the spinal cord is patterned along its antero-posterior axis through morphogen gradients and differential distribution of transcription factors that in this case mainly belong to the homeodomain factor family, namely Hox proteins. Although recent and extensive review articles have summarized most of the current knowledge on the action of Hox factors in this process [5, 48], we will briefly overview the main observations and underline the versatile, although somewhat obscure, roles of the Hox cofactors, including Foxp1, in spinal MN diversification. Combinatorial FGF, Wnt, RA, and Gdf11 signals originating from the Hensen's node and paraxial mesoderm differentially profile the expression of *Hox-c* and *Cdx* genes along the anteroposterior axis (Fig. 1a) [47, 49–51]. This indicates that morphogens shape MN diversification from the earlier steps of neural development. Progressive delivery of these extrinsic signals from more anterior to more posterior regions of the neural tube induces a delay in neuronal differentiation between rostral and caudal neurons, including MNs, which remains visible for several days. The regionalization of the expression of *Hox* genes in

response to these extrinsic signals determines the identity of the motor columns along the anteroposterior axis. Indeed, anteriorized expression of *Hox-c* genes upon induction by FGF8 in progenitor cells results in the conversion of brachial MNs into thoracic MNs [52]. Conversely, progressive inactivation of *Hox6* alleles results in gradual erosion of the brachial LMC identity [53]. Similarly, combined inactivation of *Hox10* paralog genes results in a posterior shift of the boundary between thoracic and lumbar MNs [54–56] or in an erosion of lumbar LMC identity [8], while ectopic production of *Hoxd10* in thoracic segments of chick spinal cord imposes a molecular identity and a connectivity pattern of lumbar LMC neurons [57]. Even more strikingly, inactivation of the single *Hoxc9* gene results in the transformation of most of the thoracic MNs (HMC and PGC) into neurons with the molecular identity of limb-innervating MN (LMC), and *Hoxc9* is sufficient to repress brachial *Hox* genes and LMC identity [58]. Oppositely, ectopic expression of these brachial *Hox* genes at thoracic levels promotes LMC neuron differentiation at the expense of PGC cells [53]. This further indicates that the activity of Hox proteins in patterning the spinal motor columns also relies on mutual cross-repressive interactions [52, 56, 58]. Recent data provide evidence that these interactions establish the caudal boundary of *Hox* expression domains, whereas Polycomb repressive complex 1 determines their anterior boundary through chromatin modifications [59]. Thus, the constellation of *Hox* genes and their cross-regulations establish the diversity of MNs in the different segments of the spinal cord. Whether this activity also extends to other spinal populations wherein Hox proteins are present, namely ventral or dorsal interneurons, remain to be investigated. The mechanisms that coordinate the MN anteroposterior patterning activity of the Hox proteins and the action of the proneural factors that stimulate the cell-cycle exit of the MN progenitors and the initiation of MN differentiation remains currently unknown. Of note, Hox and proneural factors synergize in *Drosophila* to stimulate the differentiation of sensory organs [60].

Hox proteins are known to require interactions with several factors to exert their activity. Different Hox cofactors have been identified in vertebrates, including members of the PBC and MEIS classes of TALE (Three Amino acid Loop Extension) homeodomain proteins and the Forkhead box p1 protein (Foxp1). The PBC class includes vertebrate Pbx homeoproteins, whereas MEIS factors include Meis and Prep homeoproteins. Pbx and Meis/Prep cofactors are able to form trimeric complexes with Hox proteins. Although initially thought to interact through the conserved hexapeptide motif of Hox factors [61–64], multiple evidence suggests that PBC and MEIS factors bind to different regions of the Hox proteins [65, 66]. Interactions with Pbx and Meis/Prep are required for Hox transcriptional activity. Nevertheless, Pbx appears to act not only as Hox cofactors

but also independently from Hox proteins to directly regulate distinct target genes and even some *HoxA/D* genes (reviewed in [63, 64]). Although they are broadly present in the developing spinal cord, the distribution of these Hox cofactors in differentiating MNs has not yet been characterized in detail [67, 68]. Whereas Pbx and Prep proteins regulate different aspects of hindbrain MN development [69, 70] and Hox/Pbx binding sites are present and required for the transcriptional activation of a 125-bp enhancer of the MN determinant gene *Hb9* [71], the roles of TALE cofactors in spinal MN development remain elusive [68] and deserve further investigation. Noticeably, recent data demonstrate that Hoxc6-Pbx interaction is required for Hoxc6 to promote brachial LMC characteristics but not for the repression of the thoracic determinant *Hoxc9* gene [53], suggesting that only part of the Hox activity in MN development may rely on TALE proteins.

In contrast, the function of *Foxp1* in MNs has been deeply characterized [8, 72, 73]. Distinct expression levels of *Foxp1* are measured in PGC and in LMC columns, and Hox proteins are required for this expression [72]. Loss- and gain-of function experiments for *Foxp1* in mouse and chick embryos suggest that these different levels in *Foxp1* are in turn necessary for proper specification of the corresponding motor columns (see below). However, these data do not implicate *Foxp1* per se in the anteroposterior patterning of the MN columns [8, 72]. Similarly, sustained Hox5 activity is required for proper development of the phrenic MNs, respiratory neurons that are generated in the rostral portion of the brachial region and provide the sole source of innervation to the diaphragm, without contribution of *Foxp1*, which is not present in these cells [74]. Thus, the exact requirement for cofactors by each Hox protein during MN development remains to be fully elucidated.

Consolidation of MN identity

Following anteroposterior patterning by the Hox proteins and activation of the proneural factors, MN progenitors exit the cell cycle and initiate neuronal differentiation, as evidenced by activation of the expression of early MN markers including *Isl1*, *Hb9*, and *Hnf-6*. However, the acquisition of this early MN identity is not persistent. Indeed, at variance to what is known for the other neuronal populations of the spinal cord, postmitotic MNs require early consolidation of their identity to maintain their fate. Defective consolidation of MN identity results in a partial or complete transformation of the MN population into dorsally adjacent V2a interneurons [75–78]. This is likely due to the fact that MNs and V2a interneurons share some of their developmental determinants, including *Nkx6* and *Lhx3* factors [79–83].

The molecular mechanisms involved in the consolidation of MN identity have been exquisitely deciphered (Fig. 2) [77, 79, 81, 83]. In the V2 domain, *Lhx3* forms a heteromeric binary complex with the nuclear LIM interactor protein NLI (Ldb, Clim), and the activity of this complex promotes V2a identity and directly stimulates the expression of V2a markers including *Chx10* [81]. This complex also prevents the activation of the MN fate-consolidating factor *Hb9* [79]. In contrast, the presence of *Isl1* and *Lhx3* in newly born MNs allows the formation of a heteromeric ternary complex with NLI [81, 83]. This homeodomain complex synergizes with the proneural bHLH factors present in MNs, including the phosphorylated form of *Neurog2* [36], to stimulate the expression of genes such as *Hb9* [36, 79, 81]. In turn, *Hb9* prevents the activation of *Chx10* while *LMO4* inhibits the formation of the V2a-specific *Lhx3*-NLI complex [77, 79]. Accordingly, inactivation of *Hb9* results in defective consolidation of MN identity and partial conversion into V2a interneurons [75, 78, 84]. Although expressed later in the course of MN differentiation, *Runx1* seems to participate in MN fate consolidation by repressing the V2 interneuron differentiation program [85], suggesting a continuous requirement for fate consolidation to maintain MN identity.

In the early phases of MN differentiation, *Hb9* indirectly stimulates *Isl1* expression [75, 78, 86, 87]. In turn, *Isl1* supports *Hb9* production, establishing a regulatory loop wherein *Isl1* and *Hb9* mutually support their expression [77, 79]. Proper control of the levels of *Isl* proteins is absolutely crucial to ensure consolidation of MN fate. Indeed, analyses of mice carrying a hypomorphic allele or a conditional inactivation of *Isl1* demonstrated that progressively reducing the levels of *Isl1* favors the differentiation of V2a interneurons at the expense of MN production [76, 77]. Consistently, constitutive inactivation of *Isl1* resulted in the complete absence of MNs at very early stages of spinal cord development [88]. This observation was attributed to increased MN cell death, pointing to a possible requirement in *Isl1* for newly born MN survival (Fig. 2). However, the generation of V2a interneurons was not investigated in these mice, hindering to formally exclude that defective consolidation of MN identity also contributed to the loss of MNs in these embryos. Although the onset of its production starts slightly later than that of *Isl1*, it seems that its paralog protein *Isl2* also participates in MN fate consolidation process. Indeed, experiments in zebrafish suggest that *Isl1* and *Isl2* have equivalent abilities to promote MN formation [89]. Furthermore, constitutive inactivation of *Isl2* in mouse increases the deficit in MN production that results from decreased *Isl1* expression [77]. Therefore, it has been proposed that the global level of *Isl* proteins, rather than a specific function attributed to each *Isl* factor, is critical for the consolidation of MN identity as well as for other

Isl-dependent processes involved in MN development [89–91]. Tbx20 may also contribute to MN consolidation by supporting in newly born MN the expression of *Isl2* and of *Hb9* [92].

Early loss of MNs in constitutive *Isl1* mutant mice and the lack of a suitable model to efficiently inactivate *Isl1* at different stages of MN differentiation prevented the identification of the time window, wherein this factor is required for the consolidation of MN identity and of later roles of Isl1 in MN development. However, recent work describing the consequences of the loss-of-function of the transcription factors of the Onecut family on MN development shed light on these questions. Onecut factors, namely Hepatocyte Nuclear Factor-6 (HNF-6), Onecut-2 (OC-2) and OC-3, are transcriptional activators present in liver, pancreas, and in the CNS during embryonic development [93–95]. These factors have been shown to regulate cell differentiation in the pancreas [96–99] and in the liver [100–102], and to control various aspects of neuronal development [103–107]. In MNs, the three OC factors are dynamically and differentially expressed in each motor column in the course of neuronal differentiation [108]. In particular, the production of HNF-6 is initiated in an “intermediate” cell population wherein Olig2 can still be detected but the early MN markers including Isl1 and Hb9 are already present, therefore corresponding to a transition state between MN progenitors and newly born MNs (Fig. 2) [9, 108]. Recently, it has been shown that OC factors directly contribute to stimulate the expression of *Isl1* during MN differentiation [9] by binding the CREST2 enhancer, which drives Isl1 production in spinal MNs [109]. In the combined absence of OC factors, the expression of *Isl1* is properly initiated in the “intermediate” population but sharply decreases in newly born and more differentiated MNs, indicating that OC factors are required to maintain *Isl1* expression in differentiating MNs. However, the amount of MNs produced in these mutant embryos was normal, as well as the amount of V2a interneurons, and no evidence of defective consolidation of the MN identity could be found. This suggests that the requirement in Isl1 for the consolidation of MN fate is restricted to the time window, wherein Isl1 is present in the “intermediate” MN population [9].

Timing of MN diversification

After initial subdivision upon the action of the Hox factors according to the segmental level they occupy along the anteroposterior axis and consolidation of their MN fate, newly born MNs undergo a diversification process that results in the production of cells displaying distinct molecular identities, migration pathways, axonal trajectories, and target connectivity located in the different motor columns

and pools. Although multiple intrinsic and extrinsic mechanisms that tightly control this process have been characterized as discussed below, the timing and the chronology of the events that segregate different MN subsets at thoracic levels of the spinal cord remains unclear.

It was initially anticipated that the segregation between somatic and visceral MN occurs before the separation between MMC and HMC neurons [75, 91]. However, BrdU incorporation experiments [110] and manipulations of the expression of motor column determinants [86] rather suggest that each subdivision is produced at the same developmental stage. Consistent with this second hypothesis, conversion of PGC neurons into HMC cells in the absence of Foxp1 [8, 72] suggests that the somatic and visceral lineages do not diverge earlier than MMC and HMC cells. In contrast, increased production of visceral MNs at the expense of somatic MNs in OC compound mutant embryos without either reduction in the total amount of MNs or modification of the MMC/HMC ratio [9], and the conversion of MMC into HMC neurons in Wnt4/5a/5b compound mutants without alteration of PGC cell number [111], rather favors the hypothesis of an initial segregation of visceral vs. somatic MN lineages followed by a subsequent separation of the MMC or HMC fates. Taking all these observations together, one may propose that the diversification of MNs in the thoracic portion of the spinal cord rely on intrinsic or extrinsic signals delivered to post-mitotic cells that retain, at least until embryonic day 10.5, enough plasticity to change their fate according to these signals. However, GDE2 modulates in a non-cell autonomous manner the differentiation potential of MN progenitors to regulate the amount of neurons in specific motor columns, namely HMC and LMC, but not in others [45]. This suggests that part of the program that determines the columnar fate of MNs, including the segregation between somatic or visceral identity, is defined at very early stages of MN development, possibly in MN progenitors. Epigenetic mechanisms activated in progenitor cells in response to the combination of intrinsic and extrinsic signals may transmit such information from MN progenitors to differentiated cells [41]. Indeed, histone H3K4 methylation is associated with active genes and, along with H3K27 methylation, is part of a bivalent chromatin mark that typifies poised developmental genes. Histone methyltransferases and histone demethylases contribute to maintain this poised state, and their inactivation alters the differentiation of progenitor cells along the neural lineage [112, 113]. Hence, these epigenetic signatures could combine to intrinsic and extrinsic cues targeting newly born MNs to eventually and permanently define the molecular identity and the columnar fate of the mature cells. Migration and axonal projection defects of differentiated MNs due to single or combined inactivation of the histone acetyltransferases CBP

and p300 are consistent with an involvement of epigenetic mechanisms in the regulation of MN diversification [41].

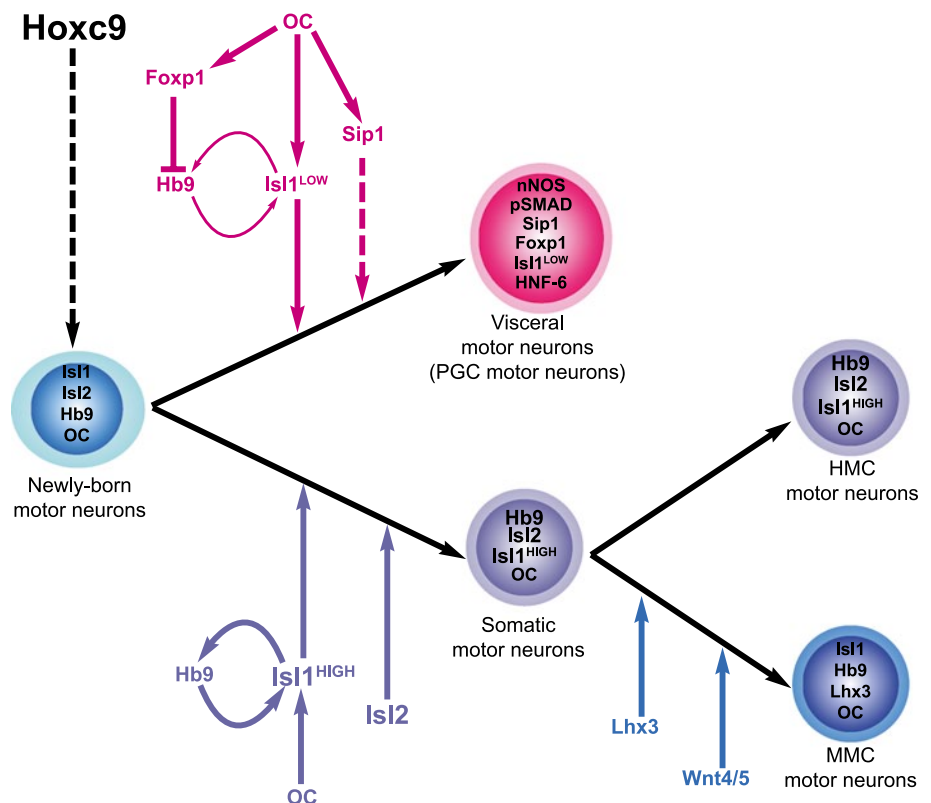
Segregation of visceral vs. somatic MN fate

One of the main divisions among spinal MNs separates somatic MNs that innervate skeletal muscles from visceral MNs that regulate the contraction of smooth muscle cells through a synaptic relay with the sympathetic neurons of the paravertebral ganglia. Different intrinsic and extrinsic factors have been involved in the separation between these two MN subsets. However, how the action of these different actors is coordinated remained elusive for a long time. The recent description of the roles of the OC factors in this process enabled to propose a comprehensive model that integrates the described functions of all these interveners (Fig. 3) [9].

Besides their early role in MN identity consolidation, Isl factors participate in the generation of visceral MNs. Indeed, constitutive inactivation of *Isl2* results in an expansion of the somatic MNs at the expense of visceral MNs at the thoracic levels of the spinal cord [91], suggesting that *Isl2* promotes the differentiation of PGC neurons. As *Isl1* and *Isl2* seem to share equivalent abilities to promote MN formation [77, 89], the role of *Isl1* in this process was also investigated. However, given that *Isl1* constitutive

mutant embryos die before the time when visceral MN can be detected, the authors relied on the observation that *Isl1* expression is severely downregulated in the absence of Hb9 [75, 78] to evaluate the contribution of *Isl1* for visceral MN production. The visceral MN differentiation defects observed in the absence of *Isl2* were indeed enhanced when *Isl1* levels were additionally reduced [91], leading to the conclusion that both Isl factors promote the production of visceral MN. However, this interpretation did not integrate a possible role for Hb9 in this process. Given that it was demonstrated later that Hb9 and Isl proteins contribute to the consolidation of MN identity (see above), alternative interpretations have been proposed for these observations [8, 9]. In addition, the Hox cofactor *Foxp1* was also demonstrated to be essential for visceral MN production. Indeed, PGC neurons contain intermediate levels of *Foxp1* while this factor is absent from the somatic MN of the MMC and HMC [72]. Ectopic production of intermediate levels of *Foxp1*, either by overexpression or by inhibition of its negative regulator miR-9, results in the expansion of the PGC neurons at the expense of their somatic counterparts, whereas *Foxp1* inactivation or miR-9 overexpression leads to a reduction in visceral MN associated with a specific expansion of the HMC neurons [8, 72, 114]. Although Isl factors and *Foxp1* very obviously participate in the segregation between somatic and visceral MN, the relationship between their respective actions remained unclear.

Fig. 3 MN diversification at thoracic levels of the spinal cord. *Hoxc9* establishes thoracic MN identity. The differentiation into somatic or visceral MN is finely tuned by the combined action of OC, *Foxp1*, Hb9, and Isl proteins. In some newly born MNs, OC factors and a mutual stimulatory loop between Hb9 and *Isl1* triggers high Isl protein levels and somatic MN differentiation. In other newly born MNs, OC stimulate *Foxp1* expression, which represses Hb9 and thereby weakens the mutual stimulatory loop between Hb9 and *Isl1* and reduces Isl levels, which favors visceral MN differentiation. OC also promote *Sip1* expression, which supports the generation of PGC cells. Among somatic MNs, *Lhx3* and activation of Wnt signaling pathway promote the differentiation of MMC neurons. The HMC fate may constitute the default differentiation fate. *nNOS* neuronal nitric oxide synthase



The recent publication of the phenotypic analysis of OC compound mutant embryos enabled to propose a model that fills this gap (Fig. 3) [9]. As previously mentioned, the absence of OC factors results in a severe reduction in the *Isl1* expression levels in newly born MNs. Although the expression of *Isl2* is not affected, this results in a strong reduction in the global levels of Isl proteins in differentiating MNs. Surprisingly, these perturbations correlate with an increased production of visceral MNs at the expense of their somatic counterpart, arguing against the hypothesis that Isl factors promote visceral MN differentiation. In addition, Isl1 protein levels in wild-type spinal cord are twice as high in prospective somatic MN than in their visceral complement, suggesting that high content in Isl proteins rather favors somatic MN fate whereas low levels would promote visceral MN production (Fig. 3). Consistently, *Isl2* overexpression (Fig. S2 in [91]) or stimulation of *Isl1* expression by overexpressing *Hb9* or the OC factor *Hnf6* in chick embryonic spinal cord decrease the amount of visceral MNs in favor of somatic MNs [9, 86, 87, 91]. Therefore, the reduction in visceral MNs observed in the absence of *Hb9* and/or Isl factors likely results from defective consolidation of MN identity rather than from a specific contribution of these factors to MN diversification [75, 77–79, 81, 91]. Indeed, MN consolidation defects result in increased production of *Lhx3* [75–78], which represses the expression of *Foxp1* [8, 72] and visceral MN differentiation [86, 91]. Hence, high contents in *Hb9* and Isl proteins would first consolidate MN identity and later promote somatic MN differentiation. In prospective visceral MNs, the presence of *Foxp1*, which is supported in these cells by OC factors [9], represses the expression of *Hb9* [8, 72]. This likely contributes to weaken the mutual stimulatory loop between *Hb9* and *Isl1*, tuning global Isl protein content to intermediate levels that are sufficient for visceral MN differentiation. Therefore, *Hb9*, *Foxp1*, Isl, and OC factors cooperate in newly born thoracic MNs to finely tune the differentiation balance between somatic and visceral MNs.

Intriguingly, the ligand BMP5 [86] and the activated form of the BMP signaling intermediates *Smad1/5/8* [72] are specifically detected in the PGC neurons from the onset of their differentiation. Moreover, *Smad*-interacting protein 1 (*Sip1*, also called *Zfhx1b* or *Zeb2*) has recently been found in visceral MNs [9]. *Sip1* is able to inhibit transcriptional activity of activated *Smad* proteins [115] but also to cooperate with the BMP-activated *Smad8* to stimulate transcription [115, 116]. Conditional inactivation of *Sip1* in differentiating MNs results in a reduction in the amount of PGC neurons, suggesting that *Sip1* promotes visceral MN differentiation [9]. Whether *Sip1* activity during MN differentiation relies on modulating BMP signaling and whether BMP5 or activation of the BMP pathway also promote PGC neuron development, require further investigations.

Recent data indicate that neonate visceral MNs can be subdivided according to their membrane properties and that these subdivisions may correspond to functionally distinct neuronal subsets [117]. Whether *Foxp1*, activated *Smad1/5/8*, *Sip1*, OC, or Isl factors, all of which are present in subsets of PGC neurons, contribute to the generation of PGC cell diversity during development remains to be established. Similarly, *Reelin* [118–120] and *Cdk5* [121] control some aspects of the migration and of the final location of the visceral MNs, but whether this additionally contributes to PGC neuron diversification is currently unknown.

Segregation of MMC vs. HMC neurons

Besides high levels of Isl proteins, *Lhx3* is a crucial determinant of somatic MN identity (Fig. 3). When overexpressed in the caudal hindbrain, it is able to convert MN with axonal projection that exit the hindbrain dorsally into MN with characteristics of spinal somatic MN including ventral axonal projections [39]. More specifically, *Lhx3* is sufficient to determine the identity of MMC neurons. Indeed, maintained expression of *Lhx3* in all the differentiating MN suppresses the production of HMC, PGC, and LMC neurons and gates MN differentiation exclusively to an MMC fate [86, 122, 123]. Surprisingly, although *Lhx3* and *Foxp1* mutually cross-repress their expression [8, 72], the loss of *Lhx3* did not result in the expansion of a *Foxp1*-dependent motor column (PGC or LMC) [39]. Oppositely, the absence of *Foxp1* at thoracic levels does not result in an increase in MMC neurons but in expansion of the HMC column at the expense of PGC cells [8, 72]. This raises the possibility that the HMC neuron identity may constitute a default pathway intrinsic to newly born MN that only requires high levels of Isl proteins to be achieved [8].

This also raises the question of the instructive factors upstream of *Lhx3* that promote MMC cell fate. Although *Lhx3* is transiently produced in all the MN at the transition between progenitors and differentiating cell, it is exclusively maintained in MMC cells at later developmental stages [39]. Wnt signaling seems to participate in this process [111]. Indeed, *Wnt4*, *Wnt5a*, and *Wnt5b* are present in the developing ventral spinal cord and cumulative analysis of their expression pattern suggests the existence of a ventral^{HIGH} to dorsal^{LOW} gradient of Wnt signaling at the time of MN differentiation. *Wnt4* and *Wnt5* overexpression favors the production of MMC neurons at the expense of their HMC counterpart, whereas progressive accumulation of *Wnt4* and *Wnt5* mutant alleles results in a progressive decreased production of MMC neurons in favor of HMC cells, without any change in PGC neurons or in the total amount of MN. Accordingly, analyzing *Nkx2.2* mutants, where the V3 interneuron progenitor domain is converted

into pMN, or chick embryonic spinal cord electroporated with a mutant isoform of the Shh receptor Smoothed that induces the conversion of the V2 interneuron progenitor domain into pMN, it was elegantly shown that ectopic ventral MN differentiate into MMC cells whereas ectopic dorsal MN rather correspond to HMC and PGC cells (Fig. 3). The role of Wnt in MN diversification does not seem to rely on the activation of the Wnt planar cell polarity pathway. Indeed, *loop tail* mice, which carry a null mutation in the *Vangl2/Ltap* gene that is essential for the transduction of this pathway, display a normal amount of MN and proportional allocation of MMC neurons [111]. Alternatively, the MMC-promoting role of the Wnt pathway might rely on a direct stimulation of *Lhx3* expression by Wnt ligands. Indeed, Wnt is able to activate *Lhx3* in neuroendocrine cells of *Ciona savignyi* embryos [124], although similar regulation in mammalian neural cells has not been demonstrated yet. Interestingly, Wnt signaling is also implicated in somatic MN fate decision in *C. elegans* [125].

Control of LMC neuron generation and diversification

The production and diversification of the LMC neurons have been investigated in great detail (Fig. 4). Indeed, the in-depth characterization of numerous motor pools and the exquisite selectivity of each of them for a specific appendicular muscle

enabled to address the whole process of neuronal development, from specification to synaptogenesis, in this single well-defined model. In the framework of this review article, we will only briefly emphasize the mechanisms that contribute to the diversity of the LMC neurons and of the motor pools. We refer to more specific reviews for a complete overview of LMC cell axonal growth and connectivity [2, 4–6].

As they specifically innervate limb and some back muscles involved in locomotion, including for example the cutaneous maximus and the latissimus dorsi [13], LMC neurons are generated in register with the embryonic limb fields. RA is the major extrinsic determinant that promotes LMC cell production at brachial level of the developing spinal cord. During embryonic development, RA is mainly synthesized by retinaldehyde dehydrogenase 2 (RALDH2) [126–129]. RALDH2 is dynamically expressed within the paraxial mesoderm directly adjacent to the spinal cord. Initially, it is found at all axial levels, but at later stages, higher levels are observed in forelimb and in hindlimb regions [127, 128, 130, 131]. Blockade of RA signaling in newly born MNs prevents brachial LMC differentiation and rather favors the production of cells displaying the characteristics of thoracic MN. Noticeably, lumbar MN do not show a similar dependence to RA, suggesting that other extrinsic factors control their production [132].

At both levels, a first wave of LMC neuron differentiation generates the prospective LMCm cells that will

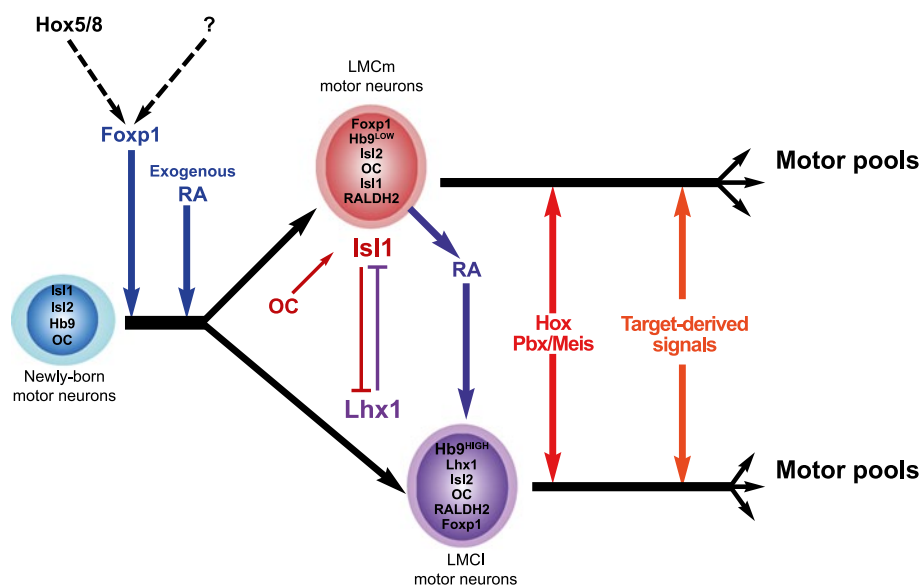


Fig. 4 MN diversification at limb levels. Exogenous RA from the paraxial mesoderm induces the generation of LMC neurons with LMCm identity. These cells rapidly initiate *Raldh2* expression and RA synthesis, which promotes the generation of a later LMC population with LMCI identity. Hox proteins, possibly generic Hox5–8 paralogs, are necessary for high *Foxp1* levels in the brachial LMC. Cross-repressive interactions between *Isl1* and *Lhx1*, respectively,

expressed by LMCm and LMCI neurons, stabilize this segregation and determine settling position and axonal projection of MNs in each division. Upon the influence of intrinsic factors, including Hox proteins and their cofactors, and of target-derived signals, subdivision of LMC neurons proceeds further and results in the formation of motor pools that individually innervate single target muscles. *RALDH2* retinaldehyde dehydrogenase 2, *RA* retinoic acid

eventually innervate the ventral complement of the appendicular musculature [132, 133]. Interestingly, these MNs rapidly activate the expression of *Raldh2* and become themselves a source of RA [134]. Subsequently, the combination of RA produced by early LMC cells [131–134] and by the nearby paraxial mesoderm [133] is necessary for a second wave of LMC neuron production that will eventually constitute the LMCI subdivision, which innervates the dorsal complement of appendicular muscles [131–134]. Interestingly, the firstly generated LMC cells do not need to acquire the specific identity of LMCm neurons for initiating *Raldh2* expression and RA production. Indeed, OC compound mutant embryos lack LMCm neurons but generate a normal amount of RALDH2-producing LMC cells that all display LMCI characteristics, including molecular identity and axonal projections [9]. This indicates that the ability to express *Raldh2* and to produce RA is a general property of LMC neurons and does not require proper columnar subdivision. Finally, RA produced by differentiated LMCm and LMCI neurons is necessary for maintenance of these cells, as MN-specific inactivation of *Raldh2* results in a global loss of LMC neurons at brachial and at lumbar levels of the spinal cord [133]. Hox factors are also involved in this maintenance process, as the expression of the RAR β receptors in the LMC requires the presence of Hoxc8 [131].

Hence, RA signaling from the paraxial mesoderm and from the LMC neurons contributes to successively generate distinct medial and lateral complements. However, additional mechanisms are required to consolidate this initial columnar division and to provide each of these complements with their own characteristics. Soon after their generation upon induction by RA, the early LMC cells initiate *Isl1* expression [135]. In these cells, *Isl1* represses the expression of *Lhx1* and determines their LMCm identity by directing their settling position in the medial portion of the LMC [136] and stimulating the expression of *EphB1*, which orients their axons towards the ventral mesenchyme of the limb buds [137]. In contrast, LMC neurons that are generated subsequently initiate the expression of *Lhx1* [135]. In these cells, *Lhx1* represses the expression of *Isl1* and imposes specific characteristics of LMCI cells including their settling position into the lateral portion of the LMC and the expression of *Epha4* and of the GDNF receptor *Ret*, which orient the axons towards the dorsal mesenchyme of the limb buds [136, 138–140]. Thus, the cross-repressive interaction between *Isl1* and *Lhx1* consolidates the columnar subdivision of the LMC initiated by RA. OC factors are required in the LMCm for the production of *Isl1*. In OC mutant embryos, LMC cells amount normally but lack *Isl1* expression and therefore adopt an LMCI identity [9]. As mentioned above, *Isl1* and *Lhx1* coordinate multiple aspects of LMC neurons

identity, including settling position and axonal projections. However, it seems that distinct downstream mechanisms control these two characteristics. Indeed, *Foxp1* and *Lhx1* cooperate in LMCI cells to maintain high levels of the Reelin signaling intermediate *Dab1*, which contributes to the lateral position of these cells within the LMC but does not impact on their axonal projection [73]. Surprisingly, expansion of the LMCI observed in OC mutant embryos did not result in a lateral displacement of the ectopic LMCI neurons [9]. This suggests that unknown effectors may intervene downstream of OC factors in this process.

Further diversification of LMC neurons divides this population into motor pools [141, 142] where each innervates a single appendicular muscle [11]. Different mechanisms contribute to the specification and to the maintenance of the motor pools, including cell-autonomous processes and target-derived extrinsic signals. Hox proteins specify the identity of distinct motor pools along the anteroposterior axis of the LMC and additionally provide an intrasegmental coding for motor pool diversity. Alterations of the Hox code result in conversions of motor pool identity and coordinated redirection of their axons towards alternative muscle target [10, 53]. Downstream of Hox factors [52], a post-mitotic period of *Nkx6.1* activity is required for the specification of the motor pools that innervate the gracilis posterior and the adductor muscles independently from target-derived signals [11]. Similarly, *PEA3* is necessary for the development of the motor pools that target the cutaneous maximus, the latissimus dorsi and the pectoralis minor muscles [13, 143]. However, activation of *PEA3* in these motor pools and proper specification of their neuronal identity require in MNs the activation of GDNF signaling by muscle-derived GDNF [144]. Subsequently, HGF signaling through its receptor *Met* is necessary in a non-cell autonomous manner for the expansion of *Pea3* expression to more anterior LMC populations [145]. Hence, the diversification of LMC neurons into motor pools is ensured by the integration of cell-autonomous and non-cell autonomous mechanisms. Although the mechanisms whereby subsets of LMC neurons that display the same molecular identity eventually gather into motor pools remain partly elusive, type II cadherins and semaphorins are specifically expressed in defined LMC pools and participate in their segregation [13, 14, 146].

Conclusions and perspectives

The quest of spinal MNs for their proper molecular identity requires passing numerous hurdles and integrating at each step intrinsic determinants with extrinsic cues produced by the nearby or more distant environment. As MNs

constitute the model of choice for studying the mechanisms that promote neuronal diversification during embryonic development, numerous molecular and cellular mechanisms involved in their differentiation have been identified. However, several aspects related to the diversification of this neuronal population into multiple subsets, the control of their migration and of their final location within the ventral spinal cord, and the exquisite manner their axon is able to find its way toward its specific muscle or sympathetic ganglion target request further investigations. In particular, the non-cell autonomous modulation of each of these processes by secreted molecules known to be expressed in the neural tube during MN development but whose influence on MN fate remains elusive would deserve additional research. Similarly, whether MNs establish crosstalks to coordinate their differentiation, their migration, or their sorting into different columns or different pools should be further investigated.

Beyond improving our basic knowledge of the mechanisms that regulate neuronal diversification, understanding the roles of the MN developmental determinants also constitutes a prerequisite for the development of cell replacement or gene therapies of MN disorders. Indeed, grafting either naive stem/induced pluripotent stem (iPS) cells or pre-differentiated cells, or modifying the genome of altered MNs or surrounding cells, can provide a therapeutic benefit in animal models of MN alterations (reviewed in [147–150]). These include models for amyotrophic lateral sclerosis [148, 150–152], spinal muscular atrophy [153, 154], spinal muscular atrophy with respiratory distress type 1 [155, 156] and traumatic spinal cord lesions (reviewed in [148]). The most critical issue regarding replacement cell therapy for MNs consists of the challenge for graft-derived or neo-generated MNs to establish innervation of peripheral targets located centimeters away from their cell body. However, experiments in mouse models established the proof-of-principle of this approach by demonstrating eventual reinnervation of skeletal muscles after MN grafting into the spinal cord [156, 157]. A deeper knowledge of the mechanisms that ensure MN diversification would benefit the three distinct aspects of these therapeutic approaches. First, it provides markers to precisely evaluate the developmental status of MN-like neurons generated from naive or predifferentiated cells grafted into an injured spinal cord. Second, it constitutes a read-out of the developmental status of stem/iPS cells undergoing in vitro differentiation into MNs [158]. Third, it generates tools to orient in vitro the differentiation of stem/iPS cells toward specific MN subsets [159, 160]. Hence, increasing our understanding of the mechanisms that regulate MN diversification should contribute to further improvement of the therapeutic approaches for MN disorders.

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