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# Transcriptional mechanisms controlling motor neuron diversity and connectivity

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The control of movement relies on the precision with which motor circuits are assembled during development. Spinal motor neurons (MNs) provide the trigger to signal the appropriate sequence of muscle contractions and initiate movement. This task is accommodated by the diversification of MNs into discrete subpopulations, each of which acquires precise axonal trajectories and central connectivity patterns. An upstream Hox factor-based regulatory network in MNs defines their competence to deploy downstream programs including the expression of Nkx and ETS transcription factors. These interactive transcriptional programs coordinate MN differentiation and connectivity, defining a sophisticated roadmap of motor circuit assembly in the spinal cord. Similar principles using modular interaction of transcriptional programs to control neuronal diversification and circuit connectivity are likely to act in other CNS circuits.

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## Introduction

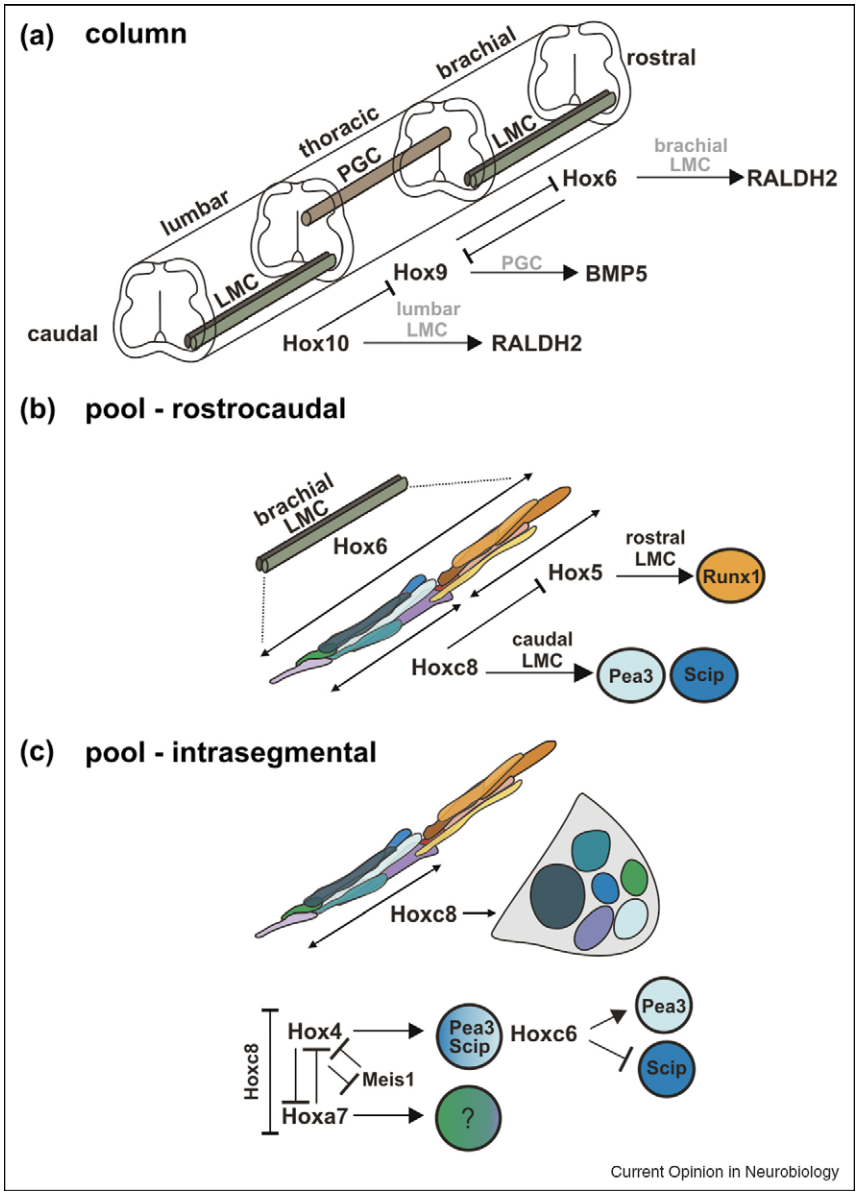
The assembly of neurons into precisely interconnected neural circuits is crucial for nervous system function and depends on the specification of defined neuronal subpopulations during development. Motor circuits are responsible for the control of movement, an animal behavior that is the final output of most nervous system activity. Initiation and execution of body movement are controlled at many levels, but all information is ultimately channeled toward motor neurons (MNs) in the spinal cord, the activation of which triggers contraction of muscles in the periphery. MNs therefore provide the exclusive action link between the nervous system and motor output. To cope with this challenging task, MN subpopulations acquire unique identities during development allowing them to receive specific connections centrally and relay this information to defined muscles peripherally.

Spinal progenitor cell differentiation is initiated by inductive signaling interactions operating through transcriptional programs [1]. As a consequence of dorsoventral signaling, MNs acquire a unique transcriptional profile (e.g. expression of the homeodomain proteins Hb9 and Isl2) distinguishing them from spinal interneuron populations shortly after leaving the cell cycle [1–4]. Recent work has begun to shed light on how distinct transcriptional networks act at postmitotic stages of differentiation to diversify MNs, the topic representing the main focus of this review. In particular, we will address how different hierarchical and parallel transcriptional programs intersect during the course of postmitotic MN differentiation to establish a unique three-dimensional motor coordinate system required to steer movement. Understanding the logic of these transcriptional control programs provides important mechanistic insights into the principles underlying the generation of a diverse array of MN subpopulations, which acquire highly specific peripheral trajectories, stereotypic cell body positions, and central connections. Moreover, we discuss how progressive transcriptional specification of MNs establishes their competence to respond to programs initiated at later differentiation steps. How these sequential genetically determined transcription factor mediated programs interact with activity-dependent processes during development has recently been reviewed elsewhere [5,6].

## Acquisition of motor neuron identity to accommodate target diversity

The establishment of precise connections between MNs and the muscular output system requires their strategic alignment along the body axis. In order to accommodate differences in peripheral targets throughout the body, MN number, identity, and connectivity differ significantly along the rostro-caudal axis of the spinal cord. Analyzed anatomically, MN cell bodies are organized into motor columns according to broad projection territories such as limbs (lateral motor column, LMC) or the autonomic nervous system at thoracic levels (preganglionic column; PGC), and these columns are formed only at appropriate segmental levels [7] (Figure 1a). Within motor columns, groups of MNs projecting to individual

**Figure 1**




Hox-mediated mechanisms controlling MN columnar and pool identities. **(a)** Schematic diagram illustrating the three-dimensional arrangement of motor columns in the spinal cord. Limb-innervating MNs are grouped in the lateral motor columns (LMCs) at brachial and lumbar levels, whereas preganglionic MNs coalesce in the preganglionic motor column (PGC) at thoracic levels. Mutually repressive interactions between Hox6 and Hox9 proteins control the development of brachial (RALDH2$^+$) and thoracic (BMP5$^+$) columnar identities in chick [14]. Hox10 paralogs direct the establishment of lumbar LMC identity [23,25,26•,29] and repress Hox9. **(b)** Diagram illustrating the Hox regulatory network controlling the emergence of MN pools along the rostro-caudal axis within the chick brachial LMC. The Hox6$^+$ brachial LMC can be subdivided into a rostral Hox5$^+$ and caudal Hoxc8$^+$ LMC domain. This rostro-caudal division constitutes an early step in the process directing transcriptional diversification of MN pool identities and is required for the control of downstream intermediate TFs such as Runx1, Pea3, and Scip in MN pools [14]. **(c)** Diagram illustrating MN pool diversification at the intrasegmental level within the caudal brachial spinal cord in chick. Multiple Hox proteins are expressed in exclusive as well as overlapping patterns, and a fine-tuned regulation of their cross-repressive interactions is required for MN pool differentiation. Within the Hoxc8 expression domain, Hox4 activity is permissive for the differentiation of both Pea3$^+$ and Scip$^+$ MN pools. The Hox4$^+$ domain is further subdivided by the expression of Hoxc6, required for the induction of Pea3 and inhibition of Scip expression [14].

muscles in the limb are clustered into MN pools, subdividing the vertebrate LMC into approximately 50 defined groups of MNs [7–10]. While many aspects of this sophisticated anatomical organization only emerge gradually during development, the initiation of phenotypical diversification is programmed into MNs at very early stages, long before motor axons first meet their target muscles.

### Hox factors and motor neuron specification

Is there a coherent molecular program that drives MNs to differentiate into distinct subpopulations at each segmental level to match the needs of their axonal targets? Several recent studies provide evidence that members of the vertebrate Hox gene family, homeodomain transcription factors consisting of 39 members located on four chromosomal clusters [11,12], play key roles in assigning both columnar and MN pool fate. In the spinal cord as elsewhere in the body, the overall expression of individual Hox genes along the rostro-caudal axis is closely linked to position within a chromosomal cluster [11,12]. This raises the possibility that the combinatorial expression patterns of Hox genes may also contribute to segmental specification of motor circuits. Analysis of Hox expression at the cellular level in combination with recently defined markers for different motor columns and MN pools has made progress possible.

How do Hox proteins program motor column and pool fate? The specification of segmentally restricted motor columns is controlled by Hox proteins with expression patterns that prefigure their rostro-caudal extents (Figure 1a). The emergence of brachial LMC MNs in the chick, marked by the expression of the retinaldehyde dehydrogenase-2 (RALDH2) gene requires Hox6 activity [13]. Similarly, at thoracic levels of the spinal cord, Hox9 activity is essential to program PGC fate characterized by the expression of bone morphogenetic protein-5 (BMP5). Specific gain- and loss-of-function experiments in the chick demonstrate that Hoxc6 and Hoxc9 activities are sufficient to transform columnar identity not only at the level of molecular markers but also with respect to the establishment of column-specific peripheral trajectories [13]. The expression patterns of other Hox proteins do not obey the rostro-caudal boundaries set by emerging columns but act in two distinct ways to specify MN pool identities [14]. MN pools occupy specific rostro-caudal subdomains within a single motor column (Figure 1b), and within the chick brachial LMC, Hox5 proteins are restricted to rostral pools while Hox8 proteins mark caudal pools [14–16] (Figure 1b). In addition, within a single rostro-caudal level, several MN pools emerge in parallel, and this intrasegmental diversification is driven by cross-repressive interactions between multiple Hox proteins [14] (Figure 1c). For example, the specification of forelimb innervating MN pools within the Hoxc8$^{+}$ domain requires the actions of Hox4, Hox6, and Hox7 paralogs [14] (Figure 1c).

Analysis of the role of Hox transcription factors in fine aspects of MN pool differentiation was heavily dependent on the availability of selective MN pool markers. Within the LMC, anatomically defined MN pools can be identified by expression of specific transcription factors, such as the ETS transcription factor Pea3 or Er81 [17,18,19••,20], the runt-related protein Runx1 [14], or the Pou-domain transcription factor Scip [14,21]. Functional experiments changing Hox codes in MN pools went hand in hand with specific alterations of MN pool markers, and these gene expression changes were tightly linked to alterations in motor axon trajectories to muscle targets [14].

Together, these findings provide strong evidence for a deterministic role of Hox networks in the diversification of MNs at columnar and pool levels. Hox factor programs play instructive roles for the acquisition of subpopulation-specific gene expression patterns and establishment of peripheral trajectories. Whether this role is also extended to the acquisition of appropriate central connectivity and maturation of late MN pool-specific traits is currently unknown. Given the dominant upstream role in programming MN identity, it is most likely that these important aspects of motor circuit assembly lie downstream of Hox transcriptional programs as well.

### Mechanisms regulating Hox activities

Are Hox gene networks used to specify all motor columns and pools? At a rough level of analysis, domains of Hox factor expression cover the entire rostro-caudal extent of the spinal cord, but their precise contribution to each segmental level has not been analyzed. At the lumbar level of the spinal cord, Hox10 proteins are expressed and play a crucial role in MN specification [22–25,26•]. However, how changes in cellular identity parallel alterations in peripheral trajectory is less clear than at brachial levels where combinatorial expression of Hox proteins at the MN pool level seems to determine target muscle innervation for at least some MN pools [14]. Moreover, it is unclear whether all MN subtypes are equally competent to respond to the activity of Hox factors or whether the cellular context programmed at progenitor stages only equips a subset of MNs to be malleable by Hox transcription factors.

It is interesting to note that MNs within the medial motor column (MMC) innervating axial muscles display differentiation properties independent of Hox activity and thus appear to escape Hox-dominated MN differentiation programs [13]. In this context, it must be considered that Hox transcription factor activity and function also depend on interaction with a variety of cofactors such as Pbx and Meis homeodomain proteins [27], some of which are expressed in subpopulations of spinal neurons during development [14,28]. Manipulation of their expression not only affects Hox target specificity, but can also result in alterations of Hox gene expression [14] and lead to phenotypes similar to mutation of Hox factors themselves [27]. Testing for degrees of Hox-responsiveness will therefore require a deeper understanding of the mechanisms through which Hox transcriptional networks regulate MN differentiation.

It is evident that the function of individual Hox proteins cannot be studied in isolation. First, Hox genes are embedded within genomic clusters and expression of at least some members is under the control of long-distance regulatory genomic elements [12]. As a consequence, altering Hox loci by mouse genetic strategies can change the expression of other Hox genes in some instances [25,29]. Functional redundancy between Hox paralogs also complicates genetic analysis in mice. By contrast, experiments in chick embryos using *in ovo* electroporation leave the genome intact and can easily assess function of paralogs [13,14]. Second, Hox proteins function through transcriptional cross-repression or activation of gene expression, and these mechanisms ensure that certain columns or MN pools are generated only at specific segmental levels [13,14]. Thus, cross-regulatory interactions between different Hox factors must be considered when studying the function of individual transcription factors. Cross-repressive transcriptional mechanisms are also used for dorso-ventral patterning at progenitor cell stages [1,30], but Hox interactions occur predominantly in postmitotic neurons [14,22]. The mechanisms by which the Hox regulatory network intersects with the output of the dorso-ventral transcriptional program(s) through which MN emerge remain to be determined. Third, Hox gene expression is also tightly regulated through histone modifications, and collinear activation of Hox genes is partly the result of a progressive opening of the chromatin structure within their genomic cluster [31]. Although epigenetic modifications are likely to play a more prominent role in the initial developmental activation of Hox loci, a complete understanding of the regulation of Hox factor activity involved in MN diversification will undoubtedly also require a comprehensive view of the epigenetic mechanisms at play at different developmental stages.

### Translating Hox activities to generate MN subtypes

At the level of MN pool specification, interactions between different Hox factors may also play an important role in assigning the neuronal number within a given MN pool, a parameter that has to be set in accordance with the target before motor axons project to the periphery. Slight alterations in cross-repressive balances may therefore be used to alter neuronal number in homologous MN pools during evolution, to parallel changes in size or identity of muscles. Comparing differences in Hox gene function in MNs innervating anterior extremities of chick (wings) and mice (forelimbs) should provide important insight into evolutionary changes and their relation to Hox transcription factor networks in the future.

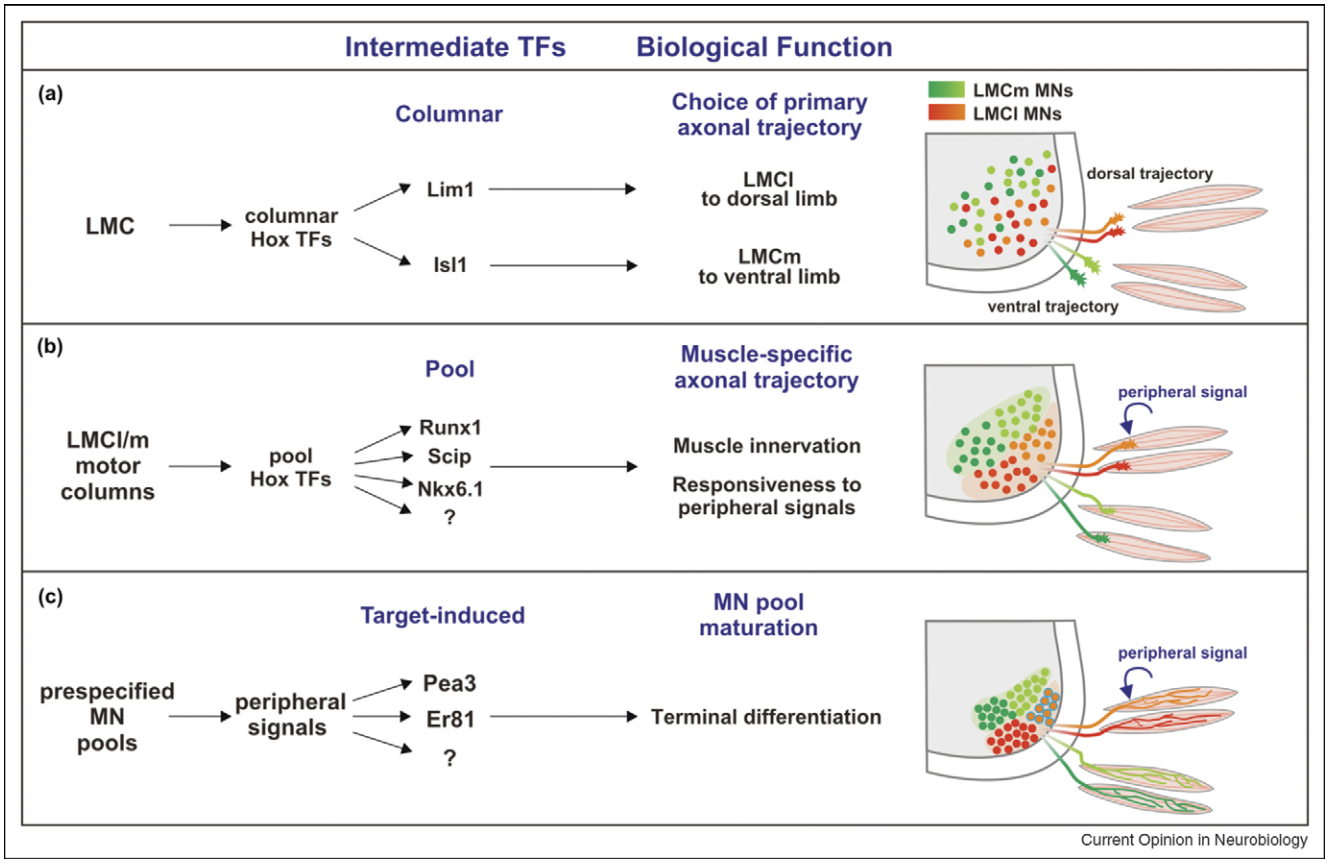
Despite the striking diversity of MN subtype identities in the spinal cord, there are also certain molecular features that are reiterated along the rostro-caudal axis. LMCs are generated at both forelimb and hindlimb levels, and these LMCs show remarkable similarities in terms of their anatomical organization and gene expression patterns. Within both brachial and lumbar LMCs, a rough topography between MN pool position and innervated muscle target exists, and MN pools innervating proximal limb muscles are found in more rostral positions than MN pools projecting to distal limb muscles [10,32–34]. Moreover, common columnar markers (e.g. RALDH2 and Lhx1) as well as MN pool markers (e.g. Pea3, Scip, Nkx6.1) are expressed by MNs within both of these columns. Despite these organizational similarities, distinct Hox regulators are involved in cervical and lumbar LMC specification, suggesting that combinatorial expression of different Hox paralogs can result in the control of both common and distinct target genes. The unique combination of Hox factors at brachial and lumbar levels thus regulates generation of MN subtypes required at each segmental level, but in addition allows implementation of essential commonalities. Although the molecular mechanism that allows distinct Hox proteins to regulate common target genes is unknown, it is likely to have its origins in the organization of the *cis*-regulatory elements within these crucial MN-specific genes.

The intersection of the transcriptional programs that determine MN specification along the dorso-ventral and rostro-caudal axes provides important information for the establishment of a three-dimensional coordinate system. These transcriptional networks could equally well be read out by other cell populations in the spinal cord and thereby contribute to diversification of interneuron subpopulations for which essentially nothing is known about possible level-specific function and connectivity. Yet, in order to ensure proper functioning of motor circuits, intersecting neuronal circuit elements such as interneurons or information provided by sensory afferents needs to be matched and coordinated with the respective local environment at a three-dimensional level. Intriguingly, Hox factors are not only expressed in distinct patterns in MNs, but also in yet undefined but restricted patterns in interneurons and dorsal root ganglion (DRG) sensory neurons [13,14]. Combinatorial profiles of Hox proteins may therefore contribute to the assembly of spinal motor circuits more generally than only in MN specification.

## Cell-intrinsic and target-induced transcriptional mediators of Hox activities

The observation that Hox factors provide important instructive cues for MN diversification raises the question of how different MNs interpret and translate combinatorial Hox expression into appropriate downstream signaling cascades and effector molecules. Recent evidence suggests that Hox transcription factors function by controlling downstream modules of intermediate transcription factors that in turn orchestrate more refined aspects of MN differentiation. These intermediate transcription factors can be grouped into a class that is cell-intrinsically

**Figure 2**




Hierarchical transcriptional programs establish MN pool identities. Illustration of transcriptional programs involved in MN specification at distinct steps of differentiation. Intrinsic or extrinsic upstream signals for individual steps **(a–c)** control the emergence of intermediate transcriptional factor (TF) programs. These programs in turn execute (a) biological function(s), gradually resulting in appropriate connectivity and function of MNs during development. Drawings to the right depict sequential MN differentiation steps at the cellular level. (a) Columnar Hox TFs direct the partition of the LMC into medial and lateral divisions through the activation of the LIM-homeodomain TFs Isl1 (LMCm; green) and Lim1 (LMCl; red). These columnar intermediate TFs determine the choice of primary axonal trajectories [7,36]. Lim1 positive MNs project to ventrally and Isl1 positive MNs to dorsally derived muscles [35]. (b) Combinatorial expression of pool Hox TFs directs the activation of pool intermediate TFs (Runx1, Scip, Nkx6.1). The expression of unique combinations of pool Hox and pool intermediate TFs controls targeting of unique peripheral muscles [40••] and equips MNs with the ability to respond to limb-derived signals. (c) Control of terminal differentiation of pre-specified MN pools is refined by target-induced intermediate TFs. Outgrowing motor axons encounter peripheral signals and MN pools respond to these cues according to their intrinsic competence and cellular context by induction of additional transcriptional programs. The activities of ETS TFs have been shown to control late aspects of MN pool maturation such as terminal MN pool positioning, axonal branching, dendritic arborization, and sensory-motor connectivity [6,18,19••].

induced through a Hox-factor-mediated program (Figure 2a and b) and a second class that requires the presence of target-derived cues (Figure 2c).

### Cell-intrinsic intermediate transcriptional programs

Expression of certain LIM-homeodomain transcription factors defines MN columnar subtypes soon after MNs become postmitotic [35,36] and some columnar expression patterns are controlled by Hox transcription factors [13] (Figure 1a). Combinatorial expression of different LIM-homeodomain transcription factors provides important cues for MN connectivity including the establishment of their initial motor axon trajectories [7,36]. For limb-innervating LMC MNs, Lhx1 instructs the outgrowth of motor axons toward dorsal limb muscles [37] (Figure 2a) and acts through the control of the Eph/Ephrin signaling system at the choice point at the base of the limb [38]. Similarly, MNs projecting toward the dermomyotome (MMCm) express Lhx3 [35] that instructs expression of the fibroblast growth factor (FGF) receptor 1, allowing these MNs to respond to target-derived FGF signals [39].

Are there similar intermediate transcription factors acting at the level of MN pools? A recent study provides evidence for a role of the homeodomain transcription factor Nkx6.1 in the establishment of MN pool specific trajectories [40••]. Nkx6.1 is transiently expressed by all MN progenitors, but its expression is rapidly restricted to MN pools at lumbar levels at the time when motor axons project toward their target muscles. Genetic experiments

support the idea that Nkx6.1 is necessary to instruct MNs innervating ventral thigh muscles to correctly grow toward their specific targets [40••]. MN pool specific expression of Nkx6.1 is independent of limb-derived retrograde signals and appears to emerge through Hox transcriptional control [40••] (Figure 2b). The cell surface molecules that may be controlled by Nkx6.1 and involved in directing axons toward their specified target muscle remain to be identified.

### Intermediate transcriptional programs induced by target-derived signals

In contrast to Nkx6.1, the expression of other MN pool specific genes is regulated by target-derived signals (Figure 2c). Two members of the ETS transcription factor family have been studied extensively (Pea3 and Er81), both with respect to regulatory pathways involved in their induction as well as function. Pea3 and Er81 are expressed in defined MN pools in the vertebrate spinal cord [17,18,19••], and limb ablation in chick embryos at early stages abolishes their induction [17]. Whereas glial cell line-derived neurotrophic factor (GDNF) induces expression of Pea3 in specific MN pools [41], the signal inducing Er81 in MNs is currently still unknown, but likely to be of mesenchymal origin [42]. *Pea3* mutant mice exhibit striking alterations in MN cell body positioning, invasion of target muscles, elaboration of MN dendrites, and sensory-motor connectivity [18,19••]. These findings demonstrate that despite the importance of early cell-intrinsic transcriptional programs for MN identity, target-derived programs also exert a profound influence on multiple aspects of MN differentiation.

How are the target-dependent programs linked to the early-established Hox transcriptional networks in MNs? As outlined above, ectopic expression of Hox genes is sufficient to reprogram MN pools and to direct peripheral axonal trajectories. This includes the induction of the ETS transcription factor Pea3 at brachial levels upon misexpression of Hoxc8 [14]. However, since Pea3 initiation requires peripheral signals [17,41], these findings suggest that Hoxc8 expression controls genes that endow MNs with the cellular competence to respond to peripheral cues encountered by Hoxc8$^+$ MNs as their axons project toward their target muscles. Consistent with this hypothesis, this domain of competence fails to be established in *Hoxc8* mutant mice and MNs cannot respond by induction of Pea3 [16].

### Establishing cellular competence and cell type specificity

These observations raise the more general question of how distinct MN pools or columns respond to similar transcriptional programs in terms of generating distinct cellular and functional output. While currently available data are still sketchy, they suggest that not all MNs respond equally to expression of the same or similar transcription factors and that the cellular context created by upstream regulatory pathways represents an important parameter in determining the output that a transcriptional network can generate. The establishment of such a cell type specific environment will ultimately depend on upstream programs, to which a variety of factors including the combinatorial activities of transcription factors, their cofactors as well as epigenetic mechanisms may contribute.

For example, whereas Nkx6.1 expression controls motor axon trajectories toward defined target muscles, mice mutant for the homologous Nkx6.2 transcription factor expressed in different MN pools do not show defects in target innervation [40••]. Similarly, while Pea3 expression in two cervical MN pools controls multiple steps in MN differentiation [18,19••], expression of the homologous transcription factor Er81 in lumbar MN pools does not influence cell body positioning or target invasion [40••]. In addition, whether MNs can respond to cues provided by the periphery with the induction of an additional transcriptional program is tightly controlled by their cellular competence. Spinal explants that are confronted with GDNF *in vitro* only induce Pea3 expression in MNs approximating the normal number *in vivo*, despite the fact that more MNs express functional GDNF receptors [41]. Moreover, heterochronic limb transplantation experiments in early chick embryos provide evidence that despite the addition of a more mature limb, the onset of ETS transcription factor expression cannot be accelerated through such manipulations [42]. Together, these findings argue that transcriptional programs create a subpopulation-specific cellular context in distinct MN pools, which allows them to read out similar signals to create different downstream outputs.

Because our knowledge of the intermediate transcriptional programs acting at the level of MN pool specification is still at an early stage, the currently available data leave open the possibility that not all MN pools depend on such programs. Some MN pools may inherit sufficient information for differentiation by directly reading out information acquired through Hox transcription factors without intersecting with additional transcriptional programs. Similarly, evolutionary expansion in the number of intermediate transcriptional programs could lead to more sophisticated differentiation programs further diversifying neuronal populations. Yet, only future work will reveal whether and to what extent the differentiation of distinct MN subpopulations occurs through a different number of intermediate transcriptional layers for different MN pools.

The concept of combinatorial cascades of transcription factors acting at distinct steps of neuronal differentiation is not unique to spinal MNs. In the DRG, sensory neuron diversification and aspects of central connectivity are

driven by activity of Runx transcription factors [6,43–45]. Moreover, the ETS transcription factor Er81 is induced by target-derived neurotrophin 3 (NT-3) in proprioceptive afferent DRG neurons [20,46], and the experiments expressing an ETS transcription regulator at premature stages of differentiation in DRG neurons suggest that the appropriate temporal activation of transcription factors is of key importance for their biological function [47]. Whether and how Hox transcription factors also prespecify DRG neurons to acquire cellular competence to downstream transcriptional intermediates is currently unknown.

## Conclusions

Collectively, the studies discussed in this review provide evidence for the existence of sophisticated transcriptional networks acting in MN subpopulations at postmitotic stages to control their appropriate differentiation and incorporation into motor circuits. The functionality of different transcription factors at sequential steps of MN maturation depends on the cellular context generated by upstream events and intersects with cues encountered by motor axons on the way to their targets. The strategy of generating distinct cellular competences by intrinsic instructive transcriptional programs allows similar permissive signaling cascades to interact with prespecified neuronal populations and yet to generate distinct outputs in different subpopulations. Owing to a stereotypic anatomical organization and easily accessible peripheral axonal trajectories, studies on MNs have been at the forefront in the identification of genetic mechanisms generating neuronal diversity. However, future work should be able to build on this knowledge to address how other neuronal subpopulations are diversified on the basis of transcriptional networks and more broadly, how specificity of connections in the nervous system is generated during development.

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