# *Hox* Genes: Choreographers in Neural Development, Architects of Circuit Organization

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**The neural circuits governing vital behaviors, such as respiration and locomotion, are comprised of discrete neuronal populations residing within the brainstem and spinal cord. Work over the past decade has provided a fairly comprehensive understanding of the developmental pathways that determine the identity of major neuronal classes within the neural tube. However, the steps through which neurons acquire the subtype diversities necessary for their incorporation into a particular circuit are still poorly defined. Studies on the specification of motor neurons indicate that the large family of Hox transcription factors has a key role in generating the subtypes required for selective muscle innervation. There is also emerging evidence that *Hox* genes function in multiple neuronal classes to shape synaptic specificity during development, suggesting a broader role in circuit assembly. This Review highlights the functions and mechanisms of *Hox* gene networks and their multifaceted roles during neuronal specification and connectivity.**

## Introduction

Nervous system development relies on the establishment of precise connections between neurons and their pre- and postsynaptic targets. In many cases the neural circuits that shape basic behaviors are defined during embryonic development, with little influence from spontaneous or sensory-evoked neuronal activity. These hard-wired programs can be linked to signaling systems operating over a narrow window during embryogenesis. A major outcome of these patterning systems is to establish specific profiles of transcription factors in neuronal progenitors and postmitotic cells, thus defining unique molecular signatures for the thousands of subtypes comprising the nervous systems of most animal species. Transcription factors orchestrate key aspects of circuit formation by deploying cell-specific programs that define the migration, projection pattern, and synaptic specificity of neuronal subtypes. A significant question is whether there are any coherent sets of developmental principles that link early progenitor identity to the incorporation of specific groups of cells into a neural circuit that controls a particular behavior.

Progress toward understanding the developmental basis of neural circuit assembly has emerged through studies on the signaling pathways that determine the identity of neuronal subtypes along the dorsoventral and rostrocaudal axes of the neural tube. The contribution of these systems to neural circuit formation has been most intensely studied along the dorsoventral axis, where each progenitor domain is specified by a unique profile of transcription factors and gives rise to distinct classes of postmitotic neurons (reviewed in Jessell, 2000; Shirasaki and Pfaff, 2002). Modern genetic tools have provided the means to assess how a single neuronal class defined by transcription factor expression contributes to circuits controlling basic motor functions (reviewed in Arber, 2012). In many cases, removal of a single transcription factor affects circuits associated with multiple motor behaviors (Bouvier et al., 2010; Lanuza et al., 2004), suggesting additional positional inputs are necessary to facilitate the incorporation of a neuron into a specific circuit.

Recent studies indicate that genetic programs acting along the rostrocaudal axis provide an important means through which neuronal classes establish subtype identities. The role of rostrocaudal positional identity in neuronal specification has been examined in the context of spinal motor neuron (MN) development, where there is a clear segregation of neurons targeting specific muscles along the rostrocaudal axis of the spinal cord (Dasen and Jessell, 2009). In addition to MNs, some of the key neural circuits controlling basic motor behaviors appear to rely on rostrocaudal positional information, including the rhythmically active circuits that control walking and breathing (reviewed in Ballion et al., 2001; Kiehn and Kjaerulff, 1998).

An important family of transcription factors that endow neural cell types with positional identities along the rostrocaudal axis are encoded by genes within the *Hox* clusters. *Hox* genes are found in all animal species and have conserved roles in body patterning (reviewed in McGinnis and Krumlauf, 1992). In most vertebrates, they are comprised of 39 genes distributed across four clusters, referred to as *HoxA*, *HoxB*, *HoxC*, or *HoxD* (Figure 1A). *Hox* genes within a cluster are classified as belonging to one of 13 paralog groups (*Hox1*–*Hox13*), and a single cluster contains only a subset of the 13 groups. Each gene is characterized by the presence of a 60 amino acid region encoding the homeodomain that mediates DNA binding. The majority of *Hox* genes are expressed in the CNS, where they have critical functions in neuronal specification and target connectivity.

In this Review, we highlight the diverse roles of Hox transcription factors in nervous system development and compare and contrast their functions in the hindbrain and spinal cord. We describe recent studies that have revealed novel strategies through which Hox proteins contribute to neuronal diversity and connectivity. Studies on the mechanisms of *Hox* gene regulation, and the pathways through which their downstream


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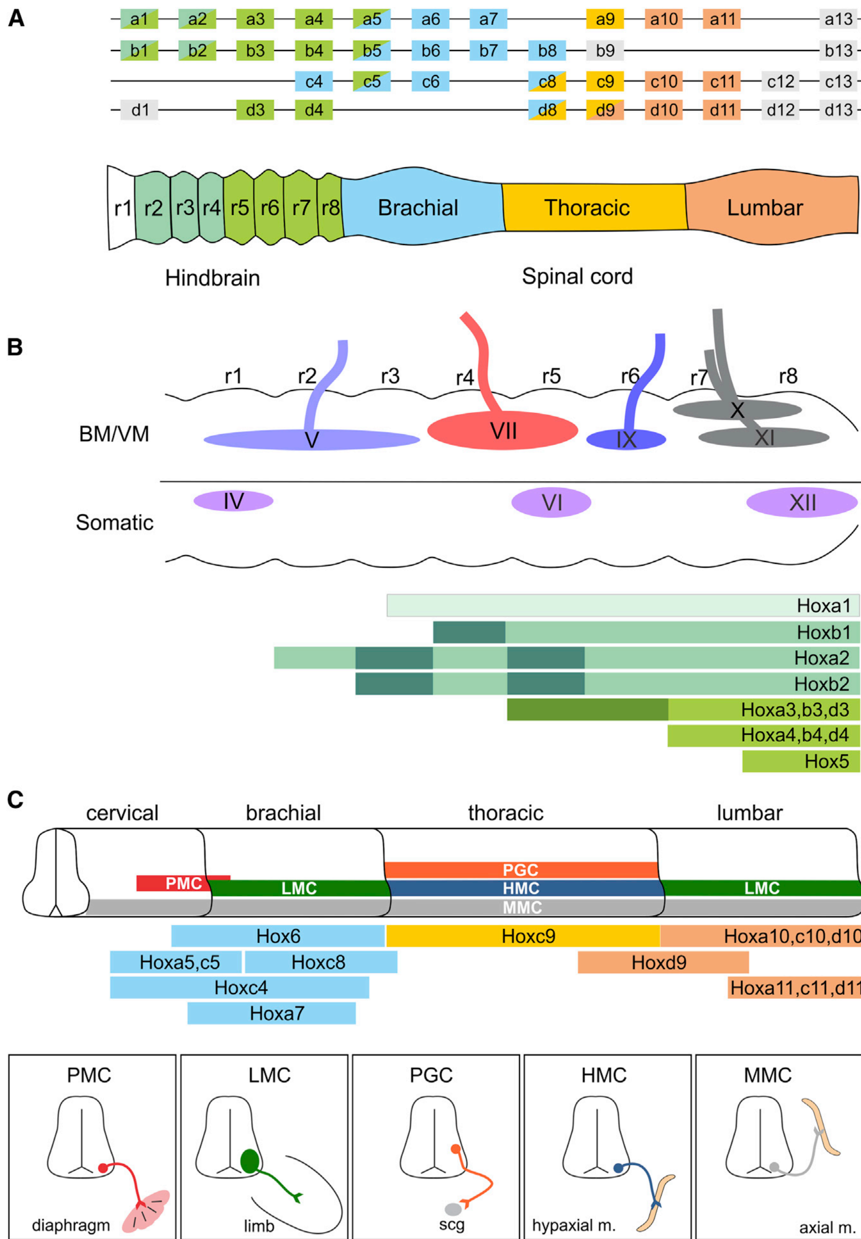


**Figure 1. *Hox* Expression Patterns in the Hindbrain and Spinal Cord**

(A) In vertebrates, 39 *Hox* genes are distributed across four clusters. Each *Hox* gene is expressed in discrete rostrocaudal domains within the hindbrain and spinal cord. Color coding of *Hox* genes represents expression domains along the rostrocaudal axis.

(B) In the hindbrain, *Hox* genes from paralog groups 1–5 are expressed and anterior expression limits correspond to rhombomere boundaries. Higher color intensity denotes higher expression. *Hoxa1* expression is transient. Hindbrain motor nuclei develop within specific rhombomeres and are shown within their rhombomeres of origin. IV, trochlear; V, trigeminal; VI, abducens; VII, facial; IX, glossopharyngeal; X, vagus; XI, accessory; XII, hypoglossal.

(C) In the spinal cord, expression of *Hox4–Hox11* genes aligns with MN columnar and pool subtypes. PMC, phrenic motor column; LMC, lateral motor column; HMC, hypaxial motor column; PGC, preganglionic motor column; MMC, medial motor column. Although technically a pool, we define phrenic MNs as a column due to their unique trajectory and because they do not reside within a larger columnar group. Peripheral targets of motor columns are indicated. LMC MNs further diversify in ~50 motor pools targeting limb muscles at brachial and lumbar levels.

effectors are controlled, have also provided important clues into how motor neuron subtypes are organized and have evolved. Moreover, recent assessments of *Hox* gene function in the hindbrain and spinal cord suggest key roles for this class of transcription factors in neural circuit assembly.

## Hox Expression and Function in the Nervous System

The embryonic hindbrain and spinal cord generate the neural circuitry required for basic motor functions such as respiration and locomotion, as well as a diverse array of sensory modalities including nociception, proprioception, audition, and balance. During development, the hindbrain is transiently segmented into eight distinct compartments, or rhombomeres (r1–r8), that give rise to the pons, medulla, and cerebellum. While no physical barriers exist between rhombomeres, differential cell adhesion properties, which develop with a conserved two-segment periodicity, prevent intermixing of cells between compartments (Guthrie et al., 1993; Wizenmann and Lumsden, 1997). Unlike the hindbrain, the spinal cord does not undergo a phase of overt segmentation; however, neuronal cell types are organized based on their rostrocaudal position. Spinal MNs, for example, exhibit stereotypical clustering patterns and follow selective axonal trajectories depending on their rostrocaudal coordinates (Landmesser, 2001). The spinal cord can be classified into cervical, thoracic, lumbar, and sacral regions, based on the segmental position of vertebrae, as well as the organization of motor and sensory nerve roots.

*Hox* gene expression in the hindbrain and spinal cord is spatially and temporally dynamic. In general, *Hox1–Hox5* paralog group genes are expressed in the hindbrain, while *Hox4–Hox11* genes are detected in the spinal cord (Figures 1A–1C). Some *Hox* genes are expressed over a narrow time window during early development, while others may persist to postnatal stages. Most of our understanding of *Hox* gene function in vertebrates derives from knockout studies in mice or manipulation of Hox activity in chick embryos. Since *Hox* genes are expressed broadly in the embryo, historically it has been

difficult to assess the cell autonomy of their function in the CNS. Moreover, even when a *Hox* gene is expressed during an early narrow time window, effects of its deletion may manifest in later steps of circuit formation, confounding the interpretation of mouse knockouts. Additional complications arise from the compensation observed between *Hox* paralog groups and cross-regulatory interactions between *Hox* genes, which result in changes in expression of other *Hox* genes in *Hox* null animals. Despite these challenges, genetic models of Hox function have provided valuable insights into their roles in CNS development (Table 1). Here we outline some of the emerging concepts from these studies as they relate to neuronal specification and circuit formation.

### *Loss of Hindbrain Compartmental Boundaries in Hox Null Mice*

In the hindbrain, initiation of *Hox* gene expression precedes segmentation, with *Hox1* paralogs, *Hoxa1* and *Hoxb1*, expressed as early as embryonic day 7.5 (E7.5), followed by paralog groups 2 and 3 at E8.5 (Tümpel et al., 2009). Mice lacking *Hoxa1* exhibit defects in hindbrain segmentation (Chisaka et al., 1992; Lufkin et al., 1991), including the absence (Carpenter et al., 1993) or reduction (Mark et al., 1993) of r5 and the reduction in the size of r4, leading to the extension of r3 (Figure 2A). While *Hoxb1* null mice do not manifest defects in early hindbrain patterning (Goddard et al., 1996; Studer et al., 1996), compound *Hoxa1/Hoxb1* mutants exhibit a more severe phenotype than *Hoxa1* mutants and lack both r4 and r5 (Gavalas et al., 1998; Rossel and Capecchi, 1999; Studer et al., 1998). *Hoxa2* mutants lack r1/r2 and r2/r3 borders (Barrow et al., 2000; Gavalas et al., 1997), leading to an expansion of the cerebellum and a corresponding reduction of pontine structures, while *Hoxa2/Hoxb2* compound mutants show a more severe phenotype, lacking interrhombomeric boundaries between r1 and r4 (Davenne et al., 1999). *Hoxa1/Hoxa2* double mutants exhibit complete absence of rhombomere boundaries (Barrow et al., 2000). It therefore appears that at the initial stages of hindbrain development, the earliest expressed *Hox1* and *Hox2* genes are responsible for its compartmentalization.

### *Hox Genes Specify the Identity and Connectivity of Cranial Nerves*

Following hindbrain segmentation, combinations of *Hox* genes act as determinants of neuronal identity within rhombomeres. In both the hindbrain and spinal cord, changes in identity and connectivity in the absence of *Hox* genes are exemplified by their effects on motor neurons (Figures 2A and 2B).

In the hindbrain, MNs are generated in specific rhombomeres and cluster in motor nuclei that can be classified as somatic, visceral (vm), or branchiomotor (bm) according to their target muscles (Guthrie, 2007). Somatic MNs are located ventrally and innervate body muscles derived from paraxial or prechordal mesoderm. Both vm and bm MNs migrate and project their axons dorsally to innervate parasympathetic ganglia and muscles in the pharyngeal arches, respectively. Hindbrain somatic MNs are derived from a progenitor domain expressing the transcription factor *Olig2*, which also gives rise to all spinal MNs, while bm and vm MNs are derived from Nkx2.2+ progenitors that give rise to V3 interneurons in the spinal cord. Despite differences in their origins, all classes of hindbrain MNs require *Hox* genes for their development.

In general, MNs that develop in regions of no or little overlap between *Hox* genes are the most susceptible to single gene mutations, likely due to lack of compensation by other paralog genes (Table 1; Figure 2A). For example, trigeminal (V) MNs develop in r2/r3, where *Hoxa2* is either the only *Hox* gene expressed (r2) or coexpressed with *Hoxb2* (r3). In the absence of *Hoxa2*, trigeminal MNs are disorganized and their axons are misrouted (Gavalas et al., 1997). Similarly, facial (VII) MNs are primarily determined by the activity of Hoxb1, which is selectively expressed in r4 (Pöpperl et al., 1995; Studer et al., 1994). In *Hoxb1*−/− mice, presumptive facial MNs acquire an r2/r3-like identity, displaying migration patterns and molecular signatures of trigeminal MNs, and fail to migrate caudally, leading to the subsequent loss of the facial nerve (Gavalas et al., 2003; Goddard et al., 1996; Studer et al., 1996). *Hoxb2*−/− and *Hoxa1*−/− mice also show a severe reduction of the facial motor nucleus, which may be partly due to their requirement for transcriptional initiation and maintenance of Hoxb1 expression (Barrow and Capecchi, 1996; Davenne et al., 1999; Gavalas et al., 2003; Helmbacher et al., 1998). Evidence that *Hox* genes act cell-autonomously in MNs is provided by misexpression studies in chick. Ectopic expression of Hoxa2 or Hoxb1 in r1 results in the generation of trigeminal- or facial-like MNs, respectively (Jungbluth et al., 1999), while Hoxb1 misexpression in r2 leads to the transformation of trigeminal to facial neurons (Bell et al., 1999). Thus, despite cooperation between *Hox* genes in multiple contexts, certain *Hox* genes also act individually to dictate specific MN identities.

While certain motor nuclei rely on the activity of single *Hox* genes, others are specified by the combinatorial expression of several homologs. For example, the abducens (VI) nucleus, containing somatic MNs originating in r5, requires the collective activity of *Hox3* group genes. These MNs are absent in *Hoxa3/b3* double mutants and can be induced by ectopic Hoxa3 expression in chick (Gaufo et al., 2003; Guidato et al., 2003). The abducens nerve is also absent in *Hoxa1*−/− mice (Mark et al., 1993), despite the transient expression of the gene prior to MN differentiation (Murphy and Hill, 1991). MNs of the glossopharyngeal (IX) nerve are derived from r6 and also require the function of *Hox3* genes for correct pathfinding (Manley and Capecchi, 1997; Watari et al., 2001). In the absence of *Hox3* paralogs, there is also a derepression of *Hoxb1* in r6, resulting in the ectopic generation and caudal migration of facial MNs (Gaufo et al., 2003). Thus, the generation of the appropriate MN subtypes in the hindbrain requires a complex interplay between multiple *Hox* genes.

### *Hox Genes in Neural Crest Cells: Non-Cell-Autonomous Effects on bm Axon Guidance*

Hindbrain branchiomotor neurons derive from multiple segments but their axons exit the hindbrain primarily from even-numbered rhombomere exit points: trigeminal (V) nerve exits from r2, facial nerve (VII) from r4, and glossopharyngeal (IX) nerve from r6 to innervate arches 1, 2, and 3, respectively. Hindbrain segmentation impinges on head development by generating neural crest cells that migrate into the pharyngeal arches. Interestingly, neural crest cells migrating to a pharyngeal arch are generated in the same rhombomeres as bm neurons innervating that arch, suggesting coordinated development of neurons and their targets

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**Table 1. Nervous System Phenotypes of *Hox* Gene Mutants**

| Mutant | Nervous System Phenotype | References |
| --- | --- | --- |
| *Hox1* genes | | |
| *Hoxa1* | Defects in caudal rhombomere boundaries, severe reduction of r4 and absence of r5, VI nerve and motor nucleus absent, smaller VII nucleus and thinner nerve with ectopic exit points, lack of superior olivary complex, defects in IX and X sensory ganglia, appearance of r2-like cells in r3, ectopic cells integrated into a novel functional respiratory network | Lufkin et al., 1991; Chisaka et al., 1992; Carpenter et al., 1993; Mark et al., 1993; Barrow et al., 2000; Helmbacher et al., 1998; del Toro et al., 2001 |
| *Hoxb1* | Homeotic transformation of r4 to r2/r3, VII MNs acquire V identity, defects in VII nucleus migration and axonal pathfinding resulting in loss of motor nucleus and nerve, misspecification of contralateral vestibuloacoustic afferents, ectopic serotonergic neuron generation in r4, loss of first-order visceral sensory neurons in r4, defects in auditory circuit formation, loss of lateral vestibulospinal tract projections | Goddard et al., 1996; Studer et al., 1996; Gavalas et al., 2003; Pattyn et al., 2003; Gaufo et al., 2004; Di Bonito et al., 2013; Chen et al., 2012 |
| *Hoxa1/Hoxb1* | Lack of rhombomeres 4 and 5, defects in patterning of cranial nerves VII through XI | Rossel and Capecchi, 1999; Studer et al., 1998; Gavalas et al., 1998 |
| *Hoxd1* | Defects in nociceptor specification and nociceptive circuit formation | Guo et al., 2011 |
| *Hox2* genes | | |
| *Hoxa2* | No r1/r2 and r2/r3 boundaries, expansion of r1-derived cerebellar territory, disorganization and abnormal pathfinding of V motor neurons, reduction of VII nucleus and nerve, defects in dorsoventral neuronal specification, loss of vestibuloacoustic afferents, loss of somatic sensory neurons in r2 and severe reduction in r3, increased oligodendrocyte production in r2/r3, defects in somatosensory map formation and sound-localizing auditory circuits | Gavalas et al., 1997; Barrow et al., 2000; Davenne et al., 1999; Gaufo et al., 2004; Miguez et al., 2012; Oury et al., 2006; Di Bonito et al., 2013 |
| *Hoxb2* | Reduced VII nucleus-partial transformation to V identity, ectopic serotonergic neuron generation in r4, decreased oligodendrocyte production in r4, defects in auditory circuit formation | Barrow and Capecchi, 1996; Davenne et al., 1999; Gavalas et al., 2003; Pattyn et al., 2003, Miguez et al., 2012; Di Bonito et al., 2013 |
| *Hoxa2/Hoxb2* | Lack of interhombomeric boundaries from r1 to r4, lack of Evx1+ interneurons in r2 and r3, defects in dorsoventral neuronal specification | Davenne et al., 1999 |
| *Hoxa1/Hoxa2* | Complete lack of rhombomere boundaries | Barrow et al., 2000 |
| *Hox3* genes | | |
| *Hoxa3* | Abnormal projections of IX and X ganglia | Manley and Capecchi, 1997; Watari et al., 2001 |
| *Hoxa3/Hoxb3* | Absence of VI nucleus, loss of first-order visceral sensory neurons in r5 | Gaufo et al., 2003, 2004 |
| *Hoxa3/Hoxd3* | Generation of VII-like MNs that caudally migrate to r7 | Gaufo et al., 2003 |
| *Hox5* genes | | |
| *Hoxa5/Hoxc5* | Abnormal diaphragm innervation, reduced and disorganized PMC | Philippidou et al., 2012 |
| *Hox6* genes | | |
| *Hoxa6/Hoxc6* | Reduction in brachial LMC | Lacombe et al., 2013 |
| *Hoxc6* | Reduction of Pea3+ pool, decreased innervation of CM muscle | Lacombe et al., 2013 |
| *Hox8* genes | | |
| *Hoxb8* | Degeneration of the second spinal ganglion, abnormal dorsal horn neuronal distribution | van den Akker et al., 1999; Holstege et al., 2008 |
| *Hoxc8* | Reduction in brachial MNs, abnormal projections into the forelimb, reduction and abnormal migration of Pea3+ motor pool | Tiret et al., 1998; Vermot et al., 2005 |
| *Hox9* genes | | |
| *Hoxc9* | Lack of PGC and HMC neurons, expansion of brachial LMC | Jung et al., 2010 |
| *Hox10* genes | | |
| Hoxc10 | Loss of lumbar MNs | Hostikka et al., 2009 |
| *Hoxa10/Hoxd10* | Loss and disorganization of lumbar MNs, defective hindlimb innervation | Wahba et al., 2001; Lin and Carpenter, 2003 |
| *Hoxc10/Hoxd10* | Acquirement of thoracic identities in lumbar MNs, abnormal hindlimb innervation | Wu et al., 2008 |
| *Hox13* genes | | |
| *Hoxb13* | Caudally extended spinal cord, supernumerary DRGs, defective tail sensory innervation | Economides et al., 2003 |

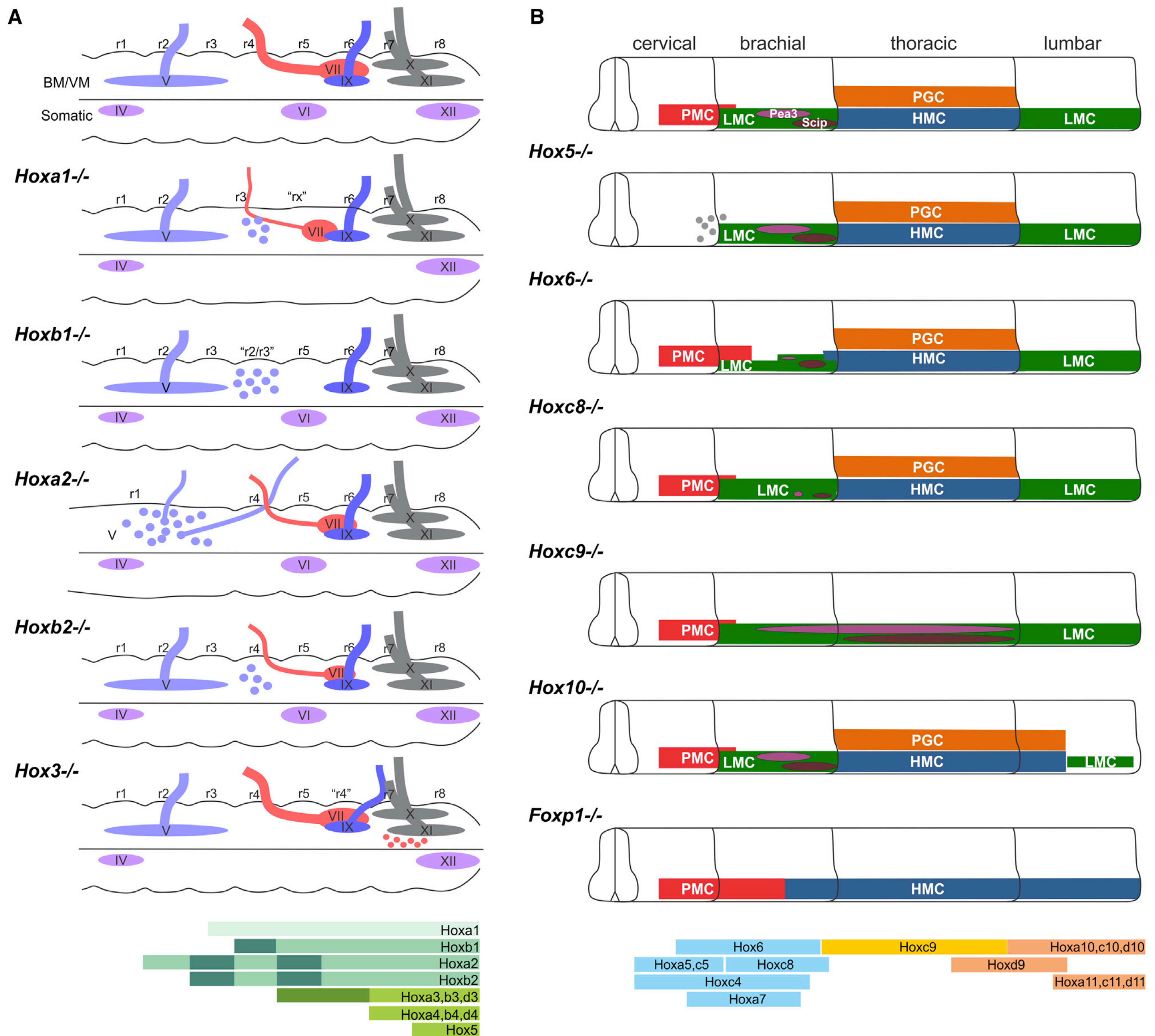


**Figure 2. Mutations in *Hox* Genes Cause Defects in MN Development, Migration, and Axon Guidance**

(A) Mutations in *Hox1–Hox3* genes result in misspecification, disorganization, and abnormal projections of hindbrain MNs. The schematics are composites, showing both early segmentation defects and subsequent MN defects. Facial MNs (VII) caudally migrate to r6 between E11–E14. The facial (VII) nucleus is absent in *Hoxb1* mutants and reduced in *Hoxa1*, *Hoxb2*, and *Hoxa2* mutants. Ectopic trigeminal nuclei are generated in *Hoxa1*, *Hoxb1*, and *Hoxb2* mutant mice but are subsequently cleared by apoptosis. Trigeminal (V) axons are misrouted in *Hoxa2* mutants and the abducens (VI) nucleus is absent in *Hoxa1* and *Hox3* mutants. In *Hoxa1* mutants, "rx" denotes a hybrid region with no clear rhombomeric identity; in *Hoxb1* mutants, r4 is transformed to r2/r3-like and in *Hox3* mutants r6 acquires an r4 identity.

(B) Mutations in *Hox5–Hox10* genes result in transformation or reduction of distinct motor columns. *Hox5* genes control PMC development, *Hoxc9* determines thoracic MN identities while *Hox6*, *Hoxc8* (at brachial levels) and *Hox10* (at lumbar levels) genes define aspects of LMC identity. *Hoxc6* and *Hoxc8* are also required for the specification of pools defined by expression of Pea3 and Scip. In *Foxp1−/−* mice, all Hox-dependent programs are disrupted with the exception of PMC specification. MMC neurons are not depicted as their development is thought to be Hox independent.

(Trainor and Krumlauf, 2001). In *Hoxa2* mutants, trigeminal MNs exit the hindbrain from r4 instead of r2, resulting in the innervation of pharyngeal arch 2 and not their correct target, arch 1. Since *Hoxa2* is also a determinant of neural crest cells migrating from r4 to arch 2, *Hoxa2* global deletion transforms the environment within arch 2 to arch 1-like (Gendron-Maguire et al., 1993; Prince and Lumsden, 1994; Rijli et al., 1993). This raises the question of whether the axon guidance defects observed are cell-autonomous for trigeminal MNs or a result of changes in environmental cues. At late stages of development, *Hoxa2* mutants also show a reduction of the facial motor nucleus and nerve, due to a requirement for *Hoxa2* in arch 2 neural crest cells

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(Briscoe and Wilkinson, 2004; Gavalas et al., 1997). *Hoxb1* expression in neural crest cells populating the second arch is also required for correct pathfinding of facial MNs, underscoring the importance of coordinated Hox activities in neurons and their target regions (Arenkiel et al., 2004). While the precise roles of *Hox* genes in the neural crest are not fully resolved, recent analysis of *HoxA* and *HoxB* cluster conditional mutants, indicates a critical cell-autonomous function in patterning of crest derivatives (Vieux-Rochas et al., 2013).

### *Hox Genes in Spinal MN Specification*

Unlike the relatively nuclear organization of MNs in the hindbrain, spinal MNs are organized into longitudinally arrayed columns and pools that span multiple segments (Figure 1C) (Landmesser, 2001). The phrenic motor column (PMC), which is unique to mammals, is generated at rostral cervical levels and innervates the diaphragm muscle. At lower cervical (brachial) and lumbar levels, MNs of the lateral motor column (LMC) project axons toward the limbs, while preganglionic (PGC) and hypaxial (HMC) neurons at thoracic levels innervate the sympathetic ganglia and hypaxial muscles, respectively. Finally, medial motor (MMC) neurons are found at all rostrocaudal levels and project dorsally to axial muscles. Forelimb and hindlimb LMC neurons further segregate in ~50 motor pools targeting specific muscles and thus comprise a highly diverse MN population.

Like hindbrain MNs, the acquisition of columnar identity in the spinal cord can require the activity of a singular or multiple *Hox* genes (Table 1; Figure 2B). The most dramatic effect of a single *Hox* gene mutation is observed in *Hoxc9* mutant mice in which thoracic motor columns acquire a brachial identity (Jung et al., 2010). *Hoxc9* mutants lack both PGC and HMC neurons and the brachial LMC extends to the anterior boundary of the lumbar LMC. This is likely due to the predominant expression of Hoxc9 at thoracic levels and the lack of compensation by other paralog genes. The ability of a single *Hox* gene to determine neuronal identity in the thoracic region also reflects the low density of expressed *Hox* genes in this region relative to limb levels (Figure 1C) (Dasen et al., 2005). A selective role for Hox proteins is evident for *Hox5* paralogs during the specification of phrenic MNs. While other *Hox* genes, such as *Hoxc6*, play an auxiliary role in setting the PMC boundaries, the absence of *Hox5* genes results in a severe defect in diaphragm innervation and a dramatic reduction and disorganization of the PMC (Philippidou et al., 2012). As a result, *Hox5* mutants perish at birth due to respiratory failure.

### *Hierarchical Roles of Hox Genes in Spinal LMC and Pool Specification*

The contributions of *Hox* genes to LMC specification reflect the varying strategies through which they contribute to MN diversification. Although *Hox6* genes are expressed by the majority of brachial LMC neurons, *Hox6* mutants still maintain an LMC, although reduced in size. Several Hox5–Hox8 paralogs can confer an LMC identity to MNs when ectopically expressed at thoracic levels of the spinal cord in chick embryos, suggesting that the early columnar identity of limb-innervating MNs is determined by redundant Hox inputs (Lacombe et al., 2013). At lumbar levels, *Hox10* genes are major determinants of LMC identity and different combinations of *Hox10* mutant alleles exhibit defects in hindlimb innervation and MN survival (Lin and Carpenter, 2003; Shah et al., 2004; Wahba et al., 2001; Wu et al., 2008). These defects are more severe than those of single *Hox10* gene mutants (Hostikka et al., 2009; Wu et al., 2008), indicating collaborative roles for *Hox10* genes in the lumbar spinal cord. Interestingly, thoracic specification programs are still suppressed in the brachial spinal cord of *Hox6* mutants (Lacombe et al., 2013), while *Hox10* mutants show a transformation of lumbar MNs to thoracic fates (Wu et al., 2008), indicating distinct mechanisms for organizing motor columns at these levels (Figure 2B).

Despite the converging actions of multiple *Hox* genes to specify LMC fate, individual *Hox* genes are required to further diversify LMC neurons into motor pools targeting specific muscles in the limb. The profile of Hox protein expression by motor pools is established through repressive interactions between *Hox* genes shortly after MNs are generated (Figure 3D). Combinatorial expression of *Hox* genes defines discrete transcriptional profiles for each pool and contributes to their clustering and peripheral synaptic specificity (Dasen et al., 2005). For example, both Hoxc8 and Hoxc6 are required for the specification of Pea3+ pools in the caudal brachial spinal cord (Figure 2B). Hoxc8 promotes specification of motor pools in the caudal half of the LMC, and in *Hoxc8* mutants the Pea3+ pool is dramatically reduced and mispositioned (Vermot et al., 2005). *Hoxc6* mutants also show a decrease of Pea3+ MNs and a severe reduction in the arborization of the cutaneous maximus muscle (Lacombe et al., 2013). Disorganization of motor pools innervating forelimb distal muscles is also observed in *Hoxc8* mutants (Tiret et al., 1998). Ectopic expression or depletion of *Hox4–Hox8* genes in the brachial spinal cord of chicks indicates that combinatorial Hox activity is critical for the establishment of pool fates (Dasen et al., 2005). Collectively, studies of *Hox* gene function in spinal cord reveal varying degrees of redundancy at the level of columnar identity but highly specific roles in motor pool specification.

### *Hox Genes and MN Generation in the Hindbrain and Spinal Cord*

While *Hox* genes have central roles in MN subtype specification in the hindbrain and spinal cord, their mode of action differs in each region. The acquisition of basic features of MN identity appears to be Hox independent in the spinal cord, established primarily as a result of dorsoventral patterning systems (reviewed in Jessell, 2000). To date, no *Hox* mutants have been reported in which spinal MN progenitors are affected, although *Hox* mutations may affect the survival or relative numbers of MNs present at brachial and thoracic levels (Dasen et al., 2008; Jung et al., 2010; Tiret et al., 1998). In contrast, manipulating *Hox* expression in the hindbrain can alter the distribution of neuronal classes specified along the dorsoventral axis (Davenne et al., 1999; Gaufo et al., 2000; Pattyn et al., 2003). In *Hoxa3/b3* compound mutants, there is a reduction of the Olig2+ MN progenitor zone and an expansion of the V2 interneuron progenitor domain (Gaufo et al., 2003). Hoxa3 overexpression in r1–r4 results in the generation of somatic MNs at the expense of V2 interneurons (Guidato et al., 2003) and both Hoxa2 and Hoxb1 can produce ectopic branchiomotor neurons when overexpressed in r1 (Jungbluth

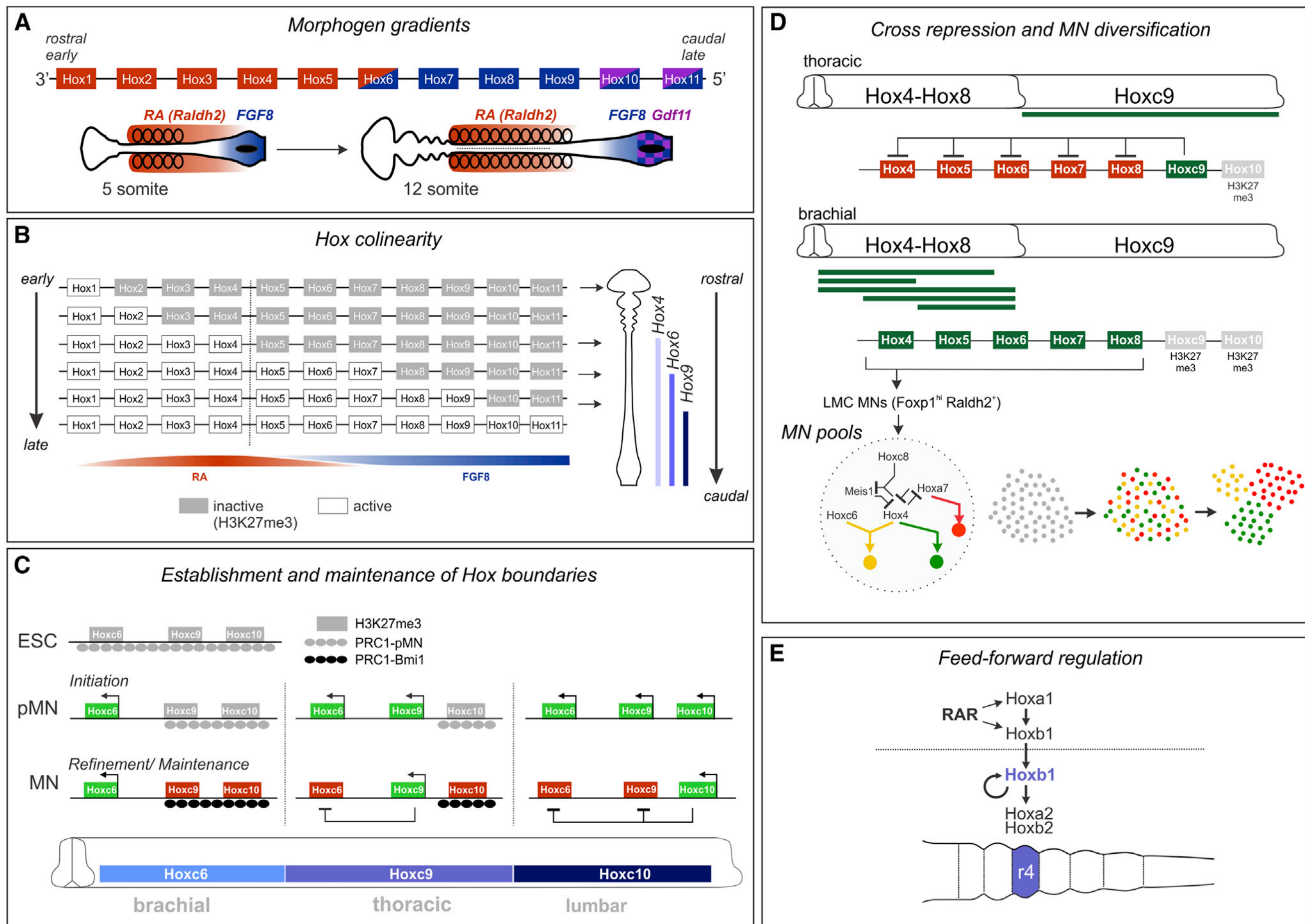


**Figure 3. Regulation of *Hox* Gene Expression in the CNS**

(A) Gradients of FGF and RA establish initial patterns of *Hox* gene expression in the early embryo. Regions of RA signaling are inferred from expression of the *Raldh2* gene in somitic mesoderm. Color coding of *Hox* genes denotes paralog groups regulated by indicated morphogens. RA induces primarily *Hox1–Hox5* genes, FGF *Hox6–Hox9*, and Gdf11/FGF8 *Hox10–Hox11* genes.

(B) Genes within a *Hox* cluster are sequentially activated along the rostrocaudal axis in a manner that is spatially and temporally linked to their chromosomal position. Colinear activation is linked to removal of repressive chromatin marks (H3K27me3). Figure on the right shows *Hox* expression is nested in more caudal regions of the embryo.

(C) Anterior limits of *Hox* gene expression are established and maintained through the actions of Polycomb repressive complexes (PRCs). In stem cells, *Hox* genes are repressed by PRCs. At the progenitor phase RA and FGF act to clear PRC2-associated methylation marks from *Hox* genes. PRC1 activities are required to maintain *Hox* gene repression in postmitotic cells. PRC1 function may be distinct in MN progenitors (pMNs, indicated in gray) as depletion of PRC1-Bmi1 only affects *Hox* expression in postmitotic MNs (Golden and Dasen, 2012). Cross-repressive effects of *Hox* genes are also indicated.

(D) Cross-repressive interactions of *Hox* genes define rostrocaudal boundaries and contribute to MN diversification. At thoracic levels, Hoxc9 excludes the expression of multiple brachially expressed *Hox* genes. At brachial levels, repressive interactions between multiple *Hox* genes determine motor pool fates. Motor pool identity appears to be specified prior to clustering, and individual MNs within a pool are indicated in yellow, green, and red.

(E) Feedforward and autoregulatory interactions among Hox1 and Hox2 paralogs ensure restricted expression of *Hoxb1* in r4. Retinoic acid receptor (RAR) mediates the activation of *Hoxa1* and *Hoxb1*. *Hoxb1* is maintained through autoregulation.

et al., 1999). It therefore appears that *Hox* genes in the hindbrain can impinge on dorsoventral fate specification programs, while no such role has yet been described in the spinal cord.

The basis for this difference may lie in the distinct temporal and spatial profiles of *Hox* genes in each region. In the hindbrain, Hox proteins are present in neural progenitors and become restricted within dorsoventral domains of specific rhombomeres (Davenne et al., 1999). This dynamic pattern allows *Hox* genes to impinge on multiple fate decisions and provides an additional layer for diversifying neuronal populations (Davenne et al., 1999; Gaufo et al., 2000). Sustained expression of *Hoxb1* in r4 mediates the prolonged generation of visceral motor neurons from a progenitor domain that switches to producing serotonergic neurons in all other rhombomeres, resulting in variations of neuronal populations within the hindbrain (Pattyn et al., 2003). In the spinal cord, Hox protein expression is predominant in postmitotic neurons, likely precluding them from influencing dorsoventral signaling pathways (Dasen et al., 2003). This difference may ultimately reflect the increased complexity of neural networks residing in the brainstem and the need for additional diversification strategies.

### *Hox Genes and Specification of Non-MN Populations in the Nervous System*

While the study of *Hox* genes in MNs has provided a valuable system to discern their functions, it is becoming apparent that other CNS populations rely on *Hox* genes for their specification (Table 1). In the hindbrain, *Hox* mutants also display defects in the formation of sensory ganglia. *Hoxb1* and *Hoxa3/b3* are required for the specification of first-order visceral sensory neurons in r4 and r5, respectively, while *Hoxa2* loss-of-function results in complete elimination of somatic sensory neurons in r2 and a severe reduction in r3 (Gaufo et al., 2004). Serotonergic neuron generation also relies on Hox-dependent programs (Pattyn et al., 2003), while *Hox2* paralog genes control oligodendrocyte production (Miguez et al., 2012). At spinal levels, *Hoxb8* has been implicated in the organization of dorsal horn neurons that relay nociceptive stimuli at lumbar levels, survival of the second spinal ganglion, and the specification of noradrenergic sympathetic neurons of the autonomic nervous system (Holstege et al., 2008; Huber et al., 2012; van den Akker et al., 1999), while *Hoxb13* acts to define the caudal boundary of the spinal cord (Economides et al., 2003).

Thus, analogous to their function in body patterning along the rostrocaudal axis, *Hox* genes act to establish segmental boundaries and regional identity within the developing hindbrain and spinal cord. A failure to establish the correct pattern of Hox protein expression in the nervous system results in changes in neuronal identity that ultimately lead to defects in axon guidance and circuit formation.

## Orchestrating *Hox* Expression in the CNS

Neuronal subtype specification in the hindbrain and spinal cord relies on Hox-dependent regionalization of progenitor and postmitotic cells along the rostrocaudal axis. Understanding how *Hox* gene profiles are established and maintained in the CNS is therefore critical in revealing how neural circuits are organized. *Hox* expression is both temporally and spatially dynamic during CNS development, involving mechanisms that are shared and distinct between the hindbrain and spinal cord.

In general, the pattern of *Hox* gene expression along the rostrocaudal axis is directly correlated with its position within the cluster, a principle termed spatial colinearity (Kmita and Duboule, 2003). *Hox* genes located at the 3′ end of a cluster are activated earlier and at more rostral levels of the neuraxis, while 5′ genes are activated later and more caudally (Figures 3A and 3B). Sequential activation of vertebrate *Hox* genes contrasts with the initiation of *Hox* gene expression in *Drosophila*, where segments form essentially in unison, and *Hox* genes are activated through the actions of segmentation transcription factors (Gellon and McGinnis, 1998). Nevertheless, in both vertebrates and invertebrates, the pattern of *Hox* expression along the rostrocaudal axis is linked to its position within a cluster, indicating that spatial colinearity is conserved among diverse species.

In broad terms, the establishment of *Hox* expression in the CNS and other tissues is defined over multiple temporally distinct phases (Figure 3). Induction of *Hox* gene expression occurs during axis extension, as stem cell-like populations emerge from the node and generate neuronal progenitors. 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). The sequential activation of genes within a *Hox* cluster is mediated by morphogens acting in a graded manner along the rostrocaudal axis. This initial pattern of *Hox* gene expression is subsequently modified through auto- and cross-regulatory interactions between Hox proteins and *Hox* genes. In general, while the initial inductive phase involves the actions of morphogens in neural progenitors, refinement and maintenance of *Hox* patterns occurs at or near the time neurons become postmitotic. Below, we review the mechanisms associated with each of these phases of *Hox* gene regulation in the hindbrain and spinal cord.

### *Morphogens and Hox Gene Colinearity*

As with patterning along the dorsoventral axis of the neural tube, the initial profile of *Hox* gene expression involves the activities of secreted morphogens acting on neural progenitors in a graded fashion (Figure 3A). The signaling pathways regulating *Hox* expression are linked to the same patterning cues involved in neural induction, and many of the signaling molecules involved in rostrocaudal patterning, notably retinoic acid (RA) and fibroblast growth factors (FGFs), also play key roles in establishing progenitor identity along the dorsoventral (DV) axis (Diez del Corral et al., 2003; Novitch et al., 2003). However, while DV patterning systems activate expression of transcription factors that are largely restricted to neural progenitors, *Hox* genes are expressed in both progenitors and postmitotic cells and are subject to distinct modes of regulation within these two cellular states.

During axis elongation, progenitor cells are exposed to graded levels of signaling molecules leading to the progressive activation of *Hox* genes located at more distal parts of the cluster. RA and FGFs exert central roles in patterning initial *Hox* expression along the neuraxis, with additional signaling systems acting to modulate these profiles (Figures 3A and 3B) (Bel-Vialar et al., 2002; Liu et al., 2001). RA provided by somites adjacent to the neural tube patterns the caudal hindbrain and rostral spinal cord. The role of RA in *Hox* regulation has been most extensively studied in the hindbrain, where it acts as a posteriorizing signal. Exposure of hindbrain progenitors to elevated RA in chick leads to an expansion of caudal rhombomeres at the expense of rostral, while inhibition of RA expands rostral and depletes caudal rhombomeres (Marshall et al., 1992). Depletion of RA signaling, through a mutation in the gene encoding the RA-synthesizing enzyme retinaldehyde dehydrogenase-2 (*Raldh2*) causes a loss of caudal *Hox* gene expression and rhombomere identity (Niederreither et al., 2000). RA promotes the expression of *Hox* genes through direct binding of retinoic acid receptors to regulatory elements in *Hox* genes, which have been characterized in *Hox1* and *Hox4* genes (reviewed in Alexander et al., 2009). RA also has an important role in patterning *Hox* expression in the spinal cord, where it regulates expression of *Hox* genes associated with rostral cervical levels (Liu et al., 2001).

FGF signaling has a key role in establishing the patterns of *Hox4–Hox10* gene expression in the spinal cord (Figures 3A and 3B). Studies in chick and embryonic stem cell-derived neuronal progenitors have shown that increasing the levels of FGF can induce *Hox* genes with a progressively more posterior

character (Liu et al., 2001; Peljto et al., 2010). Similarly, elevation of FGF signaling in vivo induces a rostral shift of *Hox* expression and transforms the identities of MN subtypes to a more caudal fate (Bel-Vialar et al., 2002; Dasen et al., 2003; Dasen et al., 2005). The effects of FGF expression in the spinal cord are mediated by Cdx homeodomain factors, as FGF can induce *Cdx* expression, and Cdx proteins are sufficient to induce expression of caudal *Hox* genes in the rostral neural tube (Bel-Vialar et al., 2002). Moreover, depletion of Cdx proteins in zebrafish confers a hindbrain identity to the spinal cord (Skromne et al., 2007), indicating that the FGF-Cdx-Hox network has a general role in distinguishing hindbrain from spinal cord neuronal identity.

FGFs also function in concert with other signaling systems to orchestrate patterns of *Hox* expression in the neural tube (Figure 3A). At rostral levels, FGF acts with RA to establish expression of *Hox6–Hox8* genes in brachial MNs. At more posterior levels, FGFs act with growth differentiation factor 11 (Gdf11) to initiate expression of *Hox10* genes at lumbar levels (Liu et al., 2001). Wnt signaling also has an obligate role in the regulation of *Hox* induction, where it specifies spinal identity and the responsiveness of progenitors to RA and FGF (Nordström et al., 2006). The establishment of graded signaling systems also relies on interactions between its primary components. For example, FGF is capable of repressing expression of *Raldh2*, thus contributing to establishing the rostrocaudal gradient of RA signaling (Diez del Corral and Storey, 2004).

### Posttranscriptional Regulation of Hox Gene Expression

The pattern of *Hox* gene expression induced by morphogens in neural progenitors is characterized by well-defined anterior boundaries, with posterior expression that often extends to the tail bud (Figure 3B). Thus, there is extensive overlap in *Hox* gene expression in progenitors at caudal levels. The specific activity of *Hox* genes in regions of nested expression has been argued to be facilitated by the ability of the more posterior *Hox* gene to suppress the activities of the anterior, a phenomenon termed posterior dominance/prevalence (Duboule and Morata, 1994). While *Hox* genes are transcribed in spinal progenitors, in many cases, Hox proteins are not observed, becoming detectable only at the time neurons differentiate (Dasen et al., 2003). Thus, the significance of posterior dominance in spinal progenitors is uncertain. While the mechanisms underlying the delay between *Hox* transcription and translation are not known, several studies indicate that *Hox* genes are posttranscriptionally regulated by both miRNAs and through translational control (Kondrashov et al., 2011; Yekta et al., 2004). These actions may serve to allow morphogens to prefigure *Hox* transcription but prevent Hox proteins from precociously activating genes that are not necessary until neurons differentiate.

### Cross-Regulatory Interactions and the Establishment of Hox Boundaries

Although nested patterns of Hox expression are observed in neural progenitors, at the time of differentiation clear posterior boundaries become apparent. The establishment of caudal boundaries has been best studied in the context of spinal MNs, where posterior limits of *Hox* expression coincide with the position of specific columnar and pool subtypes (Figure 3C). For example, the posterior boundary of Hoxc6 and Hoxc9 demarks the caudal limit of the forelimb LMC and thoracic PGC neurons, respectively, and the positional boundaries of forelimb MN pools are similarly defined by Hox expression (Dasen et al., 2003, 2005). Boundary formation is a consequence of repressive effects of Hox proteins on *Hox* genes (Figure 3D). The mechanisms mediating Hox repressive interactions have been studied in detail for the Hoxc9 protein and are facilitated by direct interactions between Hoxc9 and *Hox* genomic sequences (Jung et al., 2010). In *Hoxc9* mutants, multiple genes in the *Hox4–Hox8* paralog groups are derepressed at thoracic levels, leading to the transformation of PGC neurons to an LMC fate (Jung et al., 2010). Within LMC neurons, cross-repressive interactions among *Hox* genes contribute to the intrasegmental diversification of motor pools (Figure 3D) (Dasen et al., 2005). Similar cross-regulatory interactions appear to operate in the hindbrain, where Hox3 paralogs are required for excluding *Hoxb1* expression from r6 (Gaufo et al., 2003). However, the hindbrain appears to be less reliant on Hox cross-repressive interactions, possibly reflecting a greater dependence on molecular-based boundaries in the spinal cord, in the absence of overt segmentation.

In the hindbrain, feedforward and autoregulatory mechanisms also act to refine and maintain Hox expression, exemplified by the regulatory network that specifies the identity of rhombomere 4 (Figure 3E). *Hoxa1* and *Hoxb1* are induced in response to RA signaling prior to segmentation (Dupé et al., 1997; Studer et al., 1998), and Hoxa1 is required to maintain expression of Hoxb1 in the presumptive r4 territory (Carpenter et al., 1993). This initial pattern is reinforced through an autoregulatory enhancer in the *Hoxb1* gene, which consolidates the initial Hox input into stable Hoxb1 expression (Pöpperl et al., 1995). The pattern of Hoxb1 is further constrained to r4 through inhibitory interactions mediated by Krox20, which represses *Hoxb1* in the adjacent rhombomeres (Garcia-Dominguez et al., 2006). Hoxb1 is subsequently required to maintain expression of Hox2 paralogs in r4. The combination of positive, inhibitory, and feedforward inputs is likely to be typical of the regulatory networks that confine *Hox* gene expression in the CNS.

### Polycomb Proteins and the Refinement and Maintenance of Hox Boundaries

Classic studies in *Drosophila* indicate that the maintenance of Hox segmental boundaries is mediated by members of the Polycomb group family. Because of their critical functions in embryonic and neural stem cells, the mechanisms of Polycomb action have been intensely studied (Schuettengruber and Cavalli, 2009). In the CNS, Polycomb proteins appear to be needed for both exclusion of *Hox* expression at specific rostrocaudal positions and maintained repression in differentiated cells. In both embryonic stem cells and embryos, *Hox* clusters are characterized by a broad distribution of the repressive histone mark H3K27me3 (trimethyl-lysine-27 on histone H3), a chromatin modification associated with Polycomb repressive complex (PRC) activities (Figures 3B and 3C). During tail bud extension, H3K27me3 marks are removed from *Hox* clusters (Soshnikova and Duboule, 2009). Activation of *Hox* genes along the rostrocaudal axis correlates with the presence of the H3K4me3 chromatin mark deposited by the Trithorax complex. While it is assumed that removal of repressive marks and gain of activation marks is an obligate step in *Hox* gene activation, the precise role

of these modifications at *Hox* loci in neural progenitors is yet to be resolved.

In vertebrates, the Polycomb group encompasses a highly diverse collection of proteins with a variety of subunit compositions. Polycomb proteins form two distinct complexes: PRC2, which deposits the H3K27me3 mark, and PRC1, which recognizes H3K27me3 and mediates repression through ubiquitin ligase activity and chromatin compaction. Recent studies have challenged this canonical hierarchical view of PRC function. In stem cells lacking subunits required for the enzymatic activity of PRC2, PRC1 subunits still localize to *Hox* loci, suggesting that PRC1 can be recruited independently of H3K27me3 marks (Tavares et al., 2012). The subunit composition of PRC1 is also highly diverse, and at least six PRC1 complexes have been recognized, based on differential incorporation of Polycomb group RING finger (PCGF) family proteins (Gao et al., 2012). Some of these alternate PRC1 complexes lack the Cbx subunit that recognizes H3K27me3 and localize to *Hox* loci in cell culture models. Further investigation into the developmental roles of PRC proteins will be required to fully resolve how this network controls *Hox* gene expression in the CNS.

Recent studies have assessed the roles of PRC2 and PRC1 in CNS development. The enzymatic component of PRC2, Ezh2, is necessary for maintaining *Hox* expression in the hindbrain. Neural-specific *Ezh2* mutants are characterized by defects in pontine neuronal migration, partly as a consequence of aberrant *Hox* gene expression (Di Meglio et al., 2013). In contrast, removal of *Ezh2* from MN progenitors has no noticeable effect on Hox expression in the spinal cord (Golden and Dasen, 2012), suggesting a distinct strategy for PRC-mediated *Hox* repression. While the overall pattern of PRC2-mediated H3K27me3 occupancy at *Hox* loci appears to be determined at the progenitor phase, PRC1 function has been shown to be critical for maintaining *Hox* boundaries in postmitotic cells (Golden and Dasen, 2012). Depletion of the PRC1 component Bmi1 (PCGF4) at forelimb levels leads to ectopic expression of *Hoxc9* and the conversion of LMC neurons to a thoracic PGC fate. Conversely, elevation of Bmi1 represses *Hoxc9* at thoracic levels and converts PGC neurons to an LMC fate. These observations suggest that *Hox* repression may be maintained in MNs by distinct PRC1 activity levels along the rostrocaudal axis.

Given the importance of chromatin modifications and secreted morphogens in regulating *Hox* expression, what is the relationship between these pathways? A recent study in embryonic stem cell-derived MNs indicates a direct link; treating cells with RA depletes PRC2-associated marks from rostral *Hox* genes, while FGF in combination with Cdx2 can remove H3K27me3 from caudal *Hox* genes (Mazzoni et al., 2013). In contrast, studies in chick embryos suggest that PRC1 acts independently of the FGF8-mediated effects on chromatin status. Both elevation of FGF8 signaling and depletion of Bmi1 at brachial levels leads to identical molecular phenotypes: ectopic expression of Hoxc9, loss of Hoxc6, and a conversion of LMC neurons to a PGC fate (Dasen et al., 2003; Golden and Dasen, 2012). However, while FGF8 induces *Hoxc9* in progenitors and postmitotic MNs, depletion of Bmi1 derepresses *Hoxc9* in early postmitotic MNs, indicating that PRC1 functions in differentiated cells to maintain appropriate Hox expression patterns (Figure 3C). Collectively, these studies in stem cell-derived and embryonic MNs indicate that PRC2 and PRC1 act at distinct phases to establish and maintain the chromatin landscape of *Hox* loci.

#### Maintenance of Hox Expression in the Nervous System

Connections in the nervous system are established over the course of embryonic and postnatal development, raising the question of how long Hox actions are required within neurons. Arguably, the need for maintaining Hox expression in the CNS will rely on whether direct Hox effectors are continuously expressed and whether late expressed cell determinants require Hox function for their activities. In forelimb-innervating LMC neurons, Hoxc6 is only transiently expressed, suggesting that it only needs to be maintained over the short window necessary for it to deploy its columnar and pool program (Lacombe et al., 2013). In respiratory PMC neurons, Hox5 protein expression is extended to late embryonic phases, indicating that its maintenance is required throughout phrenic MN ontogeny (Philippidou et al., 2012). In the hindbrain, *Hox* genes are expressed up to early postnatal stages in several nuclei including the ventral cochlear nucleus and the superior olivary complex (Geisen et al., 2008; Narita and Rijli, 2009). Thus, the temporal profile of *Hox* gene expression is likely to be as important as its spatial profile for *Hox* function. Understanding the significance of *Hox* temporal regulation will require clearer knowledge about the specific pathways downstream of Hox proteins.

## Mechanisms of Hox Protein Function in the CNS

While significant progress has been made in defining the regulation of *Hox* gene expression in the CNS, the mechanisms by which they deploy cell type-specific gene programs are less well understood. Attempts to define the specificity of Hox protein function have been thwarted by two major challenges. First, Hox proteins contain conserved DNA binding domains that recognize very similar motifs (Noyes et al., 2008). Second, Hox proteins are broadly distributed among multiple classes of neurons, raising the question of how their cell-type specificity is achieved. Recent work indicates that much of the specificity of *Hox* gene function is conferred by the cofactors they associate with.

#### Hox Specificity Can Be Conferred through TALE Cofactor Interactions

Hox proteins bind AT-rich hexamer sequences through homeodomains, which are conserved among Hox paralogs (Figure 4A) (Gehring et al., 1994; Noyes et al., 2008). Thus, it has been difficult to determine how different Hox proteins accomplish specific functions, given the low selectivity in their binding motif. Hox proteins display weak affinity for DNA in vitro and high-affinity binding typically requires cooperative interaction with the TALE (three amino acid loop extension) class homeodomain proteins Pbx and Meis (reviewed in Mann et al., 2009; Moens and Selleri, 2006). Cooperative binding of Hox/TALE proteins expands the size of the DNA recognition sequence, therefore limiting the number of potential Hox targets (Figure 4A). Interactions between Hox proteins and TALE cofactors additionally enhance the binding selectivity of Hox paralogs, likely by altering their structure to facilitate contact of the N-terminal part of the homeodomain with specific DNA binding sites (Joshi et al., 2007; Slattery et al., 2011). The divergence of Hox sequences outside of the homeodomain may likewise influence Hox specificity through interactions with additional cofactors and collaborators.

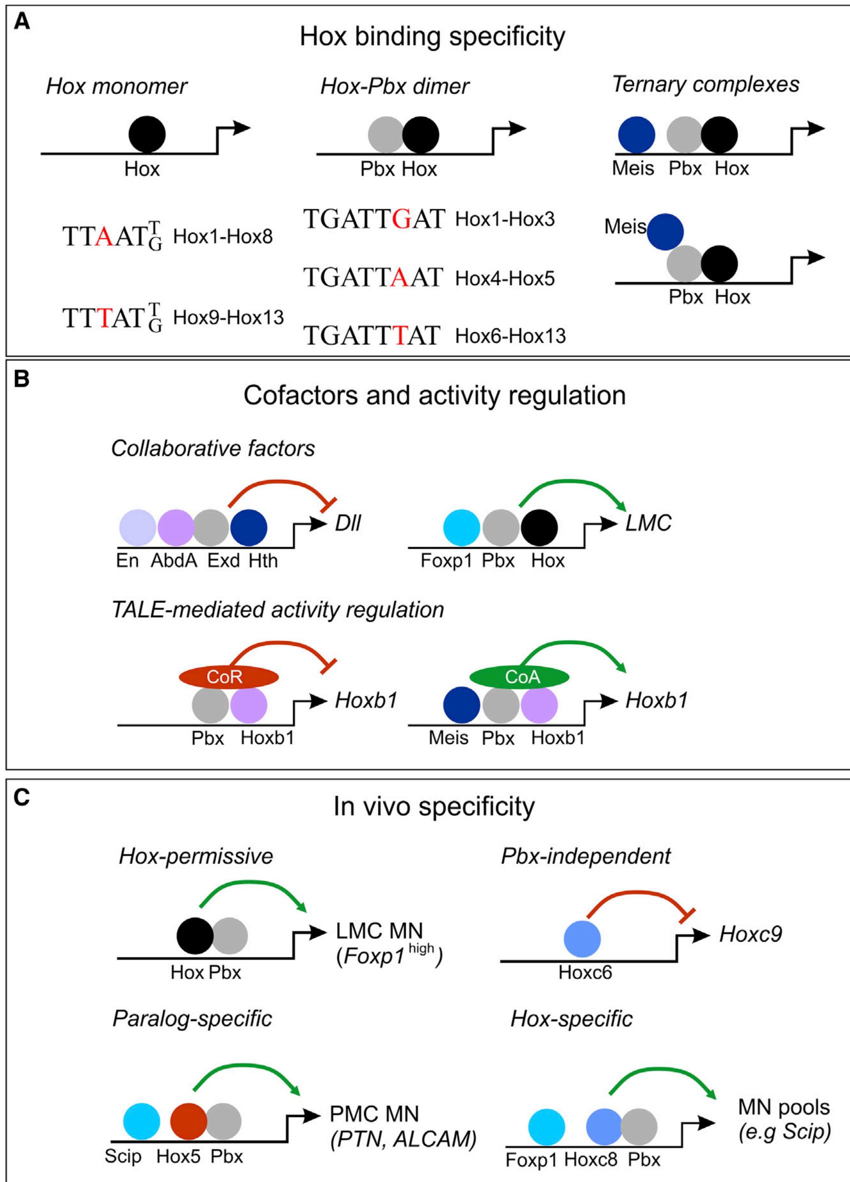


**Figure 4. Binding Specificity and Cofactors Regulate Hox Activity in the CNS**

(A) Binding of Hox proteins to Pbx cofactors enhances binding specificity to target sequences. Binding preferences for the indicated Hox paralog groups are inferred from *Drosophila* homologs (Slattery et al., 2011). Interaction of Meis proteins with Hox/Pbx dimers can occur in the absence of Meis binding sites.

(B) Cofactor interactions determine the specificity of transcriptional outputs in Hox target gene regulation. In *Drosophila*, Engrailed (En) allows the Hox protein AbdA to repress the *distalless* (*Dll*) gene. Foxp1 appears to act as a collaborative factor for many LMC-specific genes. The Foxp1/Hox interactions are speculative. At the *Hoxb1* gene, Meis proteins displace corepressors from Hox/Pbx dimers and recruit coactivators.

(C) Hox proteins display distinct specificities in determining MN subtype identities.

Due to their role as cofactors, TALE gene mutants often exhibit phenotypes similar to *Hox* mutants in the CNS. Mutations in zebrafish *Pbx* genes result in the absence of hindbrain segmentation and rhombomere specification (Pöpperl et al., 2000; Waskiewicz et al., 2002). In *Pbx2/Pbx4* zebrafish mutants, the hindbrain reverts to the r1 "Hoxless" ground state, suggesting that all Hox function in hindbrain patterning is mediated through Pbx proteins (Waskiewicz et al., 2002). However, mouse single *Pbx* mutants do not exhibit gross patterning defects in the hindbrain, which could be a reflection of more extensive redundancy among the different paralogs. *Pbx4* zebrafish mutants also show defects in the migration of the facial nucleus and the axonal pathfinding of the trigeminal nerve, although some of these phenotypes may be non-cell-autonomous (Cooper et al., 2003). In mice, *Pbx3* mutation leads to perinatal death due to central respiratory failure (Rhee et al., 2004). While the phenotype of *Pbx3*−/− mice can be partially explained by its interaction with the Tlx homeodomain transcription factor Rnx (Shirasawa et al., 2000), the involvement of several *Hox* genes in respiratory circuit formation may be indicative of multiple roles for Pbx cofactors in this process.

Meis cofactors have also been implicated in hindbrain patterning and segmentation in zebrafish (Choe et al., 2002; Deflorian et al., 2004; Vlachakis et al., 2001; Waskiewicz et al., 2001), although CNS defects in *Meis* mouse mutants have not been reported (Azcoitia et al., 2005; Hisa et al., 2004). Interestingly an important function of Meis proteins in the hindbrain is to displace corepressors from Pbx proteins and recruit coactivators (Figure 4B) (Choe et al., 2009). Therefore, it is possible that the identity of the TALE cofactor incorporated into a Hox complex may play an additional role in gating its activity. In mice, there are four Pbx and five Meis homologs and whether these have different affinities for different Hox proteins or other cofactors remains to be determined.

Not all Hox binding to target sequences is dependent on TALE cofactor interactions, and it appears that there is a bias toward repressing, as opposed to activating, gene targets when these cofactors are absent (Mann et al., 2009). Studies in *Drosophila* indicate that monomeric binding of Hox proteins represses transcription (Galant et al., 2002). Consistent with this idea, mutation of the Pbx-interaction motif in the Hoxc6 protein eliminates its cooperativity with Pbx3 but preserves its ability to repress the *Hoxc9* gene (Lacombe et al., 2013). However, Hox/TALE interactions can also mediate repression of target genes. For example, the *Drosophila* homeodomain protein Engrailed (En) is a Hox cofactor that mediates repression of the target gene *Distalless*

in a TALE-interaction-dependent manner (Figure 4B) (Gebelein et al., 2004). These observations indicate that the output of Hox/TALE interactions may rely on the additional cofactors it interacts with, rather than intrinsic transcriptional activities. It should also be noted that TALE proteins can play roles independent of Hox proteins, exemplified by the role of *Drosophila* Homothorax in antennae development, a tissue lacking any *Hox* gene expression (Casares and Mann, 1998).

#### *Hox Activities Shared and Unique among Paralogs*

A common theme from studies of Hox specificity in the hindbrain and spinal cord is the regulation of both converging and diverging pathways by Hox proteins. Several Hox functions appear to be shared between multiple Hox proteins, most frequently paralogs, while others are unique to a single Hox protein (Figure 4C). For example, in spinal MNs, multiple genes in *Hox5–Hox8* paralog groups contribute to LMC identity at brachial levels, while Hoxc9 uniquely confers thoracic PGC identity (Jung et al., 2010; Lacombe et al., 2013). Among paralog groups, Hoxa5 and Hoxc5 cooperate to control PMC development (Philippidou et al., 2012). In the hindbrain, expressing Hoxa1 from the *Hoxb1* locus can rescue facial nerve defects in *Hoxb1* mutant mice, despite diverse functions of the two proteins in vivo (Tvrdik and Capecchi, 2006). Conversely, individual Hox proteins, such as Hoxc6 and Hoxc8, control unique aspects of MN pool identities (Dasen et al., 2005; Lacombe et al., 2013).

At the level of target gene regulation, Hox proteins collectively determine the migration, survival, and guidance of neuronal subtypes, possibly by activating common or related downstream targets. The acquirement of different identities and trajectories upon ectopic expression of Hox proteins indicates that there is also divergence of targets that confer specific neuronal identity. It would then appear that multiple Hox proteins could share common effectors but also that each Hox protein would have a unique set of targets to further fine-tune neuronal identity. The convergence of Hox proteins on a common set of targets could also provide a mechanism through which variation in Hox protein levels or activity could determine the relative expression of a particular gene. In MN axon guidance, for example, the relative rather than absolute expression level of guidance receptors such as RET, GFRa1, and ephrins appears to be important for correct pathfinding (Bonanomi et al., 2012). Ephrins are among the known Hox target genes and therefore it is plausible that differential efficacies of Hox-mediated gene activation may regulate ephrin levels.

### Effectors of Hox Protein Activities in the CNS

While the pathways acting downstream of *Hox* genes are at present not well-defined, several studies in the hindbrain and spinal cord indicate that Hox proteins deploy a variety of intermediate transcription factors that in turn activate signaling pathways that contribute to neuronal connectivity. In some contexts, Hox proteins may bypass intermediate targets and directly activate specificity determinants.

#### *Transcription Factors Functioning Downstream of Hox Proteins*

Several factors acting downstream of *Hox* genes have been identified that mediate subsets of Hox functions in the CNS (Figure 5). In the hindbrain, mutations in *Hox1* and *Hox2* genes affect the expression of factors involved in cell-type specification, such as Phox2b, Nkx proteins, and Pax6 (Davenne et al., 1999; Gaufo et al., 2000; Pattyn et al., 2003). *Phox2b*, a determinant of cranial bm and vm neuron specification, is a direct target of Hoxb1 and Hoxb2 (Samad et al., 2004). GATA2 and GATA3 act downstream of Hoxb1 to control facial MN migration and contralateral vestibuloacoustic efferent neuron projections in r4 (Pata et al., 1999). The interneuron determinant *Evx1* is regulated by Hox2 paralogs, while *Hox3* genes confer somatic MN identity by upregulating *Olig2* and *Hb9* (Davenne et al., 1999; Gaufo et al., 2003; Guidato et al., 2003).

In the spinal cord, a major target effector of Hox proteins in MNs is the gene encoding the transcription factor Foxp1 (Dasen et al., 2008; Rousso et al., 2008). Limb-level Hox proteins induce high levels of *Foxp1* to specify LMC fates, whereas Hoxc9 induces low levels of *Foxp1* at thoracic levels to specify PGC fates. Hox-dependent regulation of *Foxp1* determines columnar identity, as elevation of Foxp1 at thoracic levels can convert PGC and HMC neurons to an LMC fate. Once induced, Foxp1 also acts as an accessory factor for *Hox* genes and is required for the expression of all LMC motor pool determinants (Dasen et al., 2008). In *Foxp1* mutants, there is a loss of columnar and pool identities and a randomization of axonal projections and cell body position, likely reflecting loss of downstream effectors such as ephrins and cadherins (Figures 5A and 5B). Within LMC neurons Hox proteins also coordinate the expression of multiple pool-restricted transcription factors. The transcription factors Nkx6.1, Pea3, and Scip are deployed downstream of *Hox* genes and act to specify facets of identity within LMC pools. In the absence of *Nkx6.1*, axons from lumbar MN pools fail to innervate their appropriate muscle targets (De Marco Garcia and Jessell, 2008), while *Pea3* mutation leads to defects in MN clustering and intramuscular branching (Figure 5B) (Livet et al., 2002). The extent of cooperativity between *Hox* genes and these downstream factors also remains to be determined and will heavily depend on the identification of the final effector targets in these cascades.

While the diversification of LMC neurons relies on multiple *Hox* genes and downstream effectors, some inroads into deciphering target gene regulation can be made by analyzing populations that rely on a single *Hox* paralog group. The development of phrenic MNs is determined by the *Hoxa5* and *Hoxc5* genes (Philippidou et al., 2012). In *Hox5* mutant mice, multiple aspects of PMC identity are compromised, including cell body clustering, axon guidance, intramuscular branching, and survival (Figure 5C). Late removal of *Hox5* genes from postmitotic MNs demonstrated that prolonged Hox5 expression is necessary for maintenance of certain target genes, such as the trophic factor *pleiotrophin* (*PTN*). *PTN* is under direct regulation by Hoxa5 (Chen et al., 2005), indicating that Hox proteins can act to induce effector molecules directly and not exclusively through intermediate factors. PMC neurons also express the transcription factor Scip (Pou3f1); however, expression of Scip appears to be incapable of conferring a PMC identity in the absence of *Hox5* genes. Since Pou proteins have been shown to bind DNA collaboratively with Hox proteins (Di Rocco et al., 2001), it is possible that Hox5 and Scip have a synergistic rather than a linear relationship in regulating PMC specific genes.

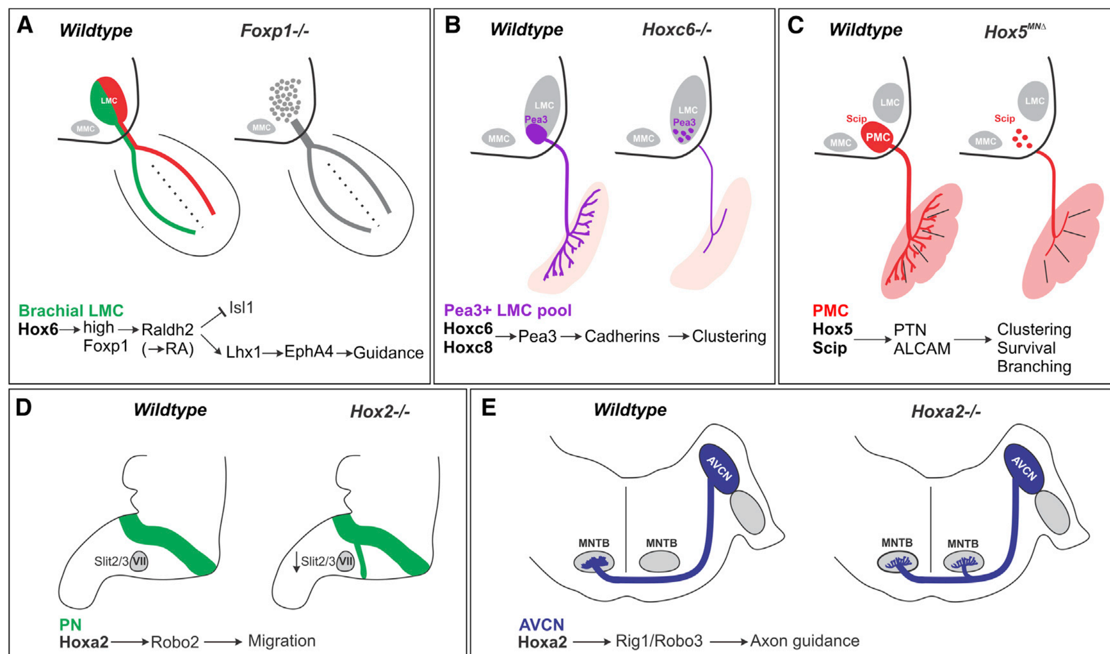


**Figure 5. Hox Effectors Control Aspects of Subtype-Specific Neuronal Identity and Connectivity**

(A) LMC neurons are disorganized and project haphazardly in *Foxp1−/−* mice, due to the loss of adhesion and guidance molecules, such as EphA4.

(B) *Hoxc6−/−* and *Hoxc8−/−* mice have a reduced and disorganized Pea3+ pool, due to loss of cadherin expression.

(C) MN-specific *Hox5* deletion results in defects in multiple aspects of PMC development, likely due to downregulation of target effectors such as *ALCAM* and *PTN*.

(D) Tangentially migrating pontine neurons (PNs) express Robo2, which prevents premature ventral migration by responding to Slit2/3 repulsive signals secreted from the facial motor nucleus (VII). In the absence of *Hox2* genes, both *Robo2* and *Slit2/3* are downregulated, resulting in abnormal pontine neuron migration.

(E) *Hoxa2* inactivation perturbs anteroventral cochlear neuron (AVCN) axonal pathfinding to the medial nucleus of the trapezoid body (MNTB) in the superior olive, resulting in decreased contralateral and increased ipsilateral targeting of MNTB due to the downregulation of *Rig1/Robo3*, the main axon guidance receptor required for midline crossing.

### *Guidance, Adhesion, and Migratory Pathways Regulated by Hox Proteins*

While *Hox* genes control a diverse array of cellular processes, there is evidence indicating that they can achieve specific outcomes by regulating the same effector classes in different contexts. One class of Hox target effectors belong to the Eph/Ephrin receptor and ligand system. During early stages of hindbrain compartmentalization, Eph/ephrin-mediated attractive and repulsive interactions act to maintain rhombomere boundaries, with receptors and ligands expressed in alternate rhombomeres. One Eph receptor, *EphA2*, is under direct regulation of Hox1 paralogs (Chen and Ruley, 1998), while *EphA4* and *EphA7* are downregulated in r2 and r3 of *Hoxa2−/−* mice, respectively (Gavalas et al., 1997; Taneja et al., 1996), indicating that regulation of Eph receptors by Hox proteins contributes to rhombomere segregation. Hoxa2 may also employ the Eph/ephrin signaling system in defining topographic connectivity of the rostral principal (PrV) nucleus to the thalamus by regulating expression of *EphA4* and *EphA7* (Oury et al., 2006).

In spinal MNs, Eph/ephrin signaling has a critical role in the guidance of LMC axons, in particular their initial choice between a ventral and dorsal trajectory at the base of the limb bud. Hox proteins in LMC neurons regulate expression of Raldh2, which provides an MN-derived source of RA that induces expression of the transcription factor *Lhx1* (Figure 5A). Lhx1 in turn stimulates *EphA4* expression, which is necessary for defining the dorsal trajectory of lateral LMC neurons (Kania and Jessell, 2003). While regulation of EphA4 in MNs is indirect, *Hox* genes could also have a more direct role, as multiple Eph and Ephrin family members are expressed and function in several MN subpopulations (Feng et al., 2000; Iwamasa et al., 1999).

Besides the ephrins, other axon guidance systems have been shown to be regulated by Hox proteins. The Robo/Slit receptor/ligand system mediates repulsive cell interactions. In *Hox2* mutants, *Robo2* is downregulated in migrating pontine neurons, contributing to their abnormal migration and premature attraction toward the midline (Figure 5D). Interestingly, in these mutants, *Slit2/3* are also downregulated in the facial motor nucleus, the source of repulsive cues, exacerbating the phenotype (Geisen et al., 2008). Hoxa2 also directly regulates the guidance receptor *Rig1/Robo3*, which controls the trajectory of a subset of cochlear neurons in the hindbrain (Figure 5E) (Di Bonito et al., 2013). Robo/Slit signaling contributes to establishing appropriate patterns of intramuscular branching of spinal MNs (Jaworski and Tessier-Lavigne, 2012); however, whether this system is under Hox regulation in the spinal cord has not been explored. Another receptor mediating repulsive responses to netrin, *Unc5b*, appears to be repressed by Hox5 proteins during pontine neuron migration (Di Meglio et al., 2013).

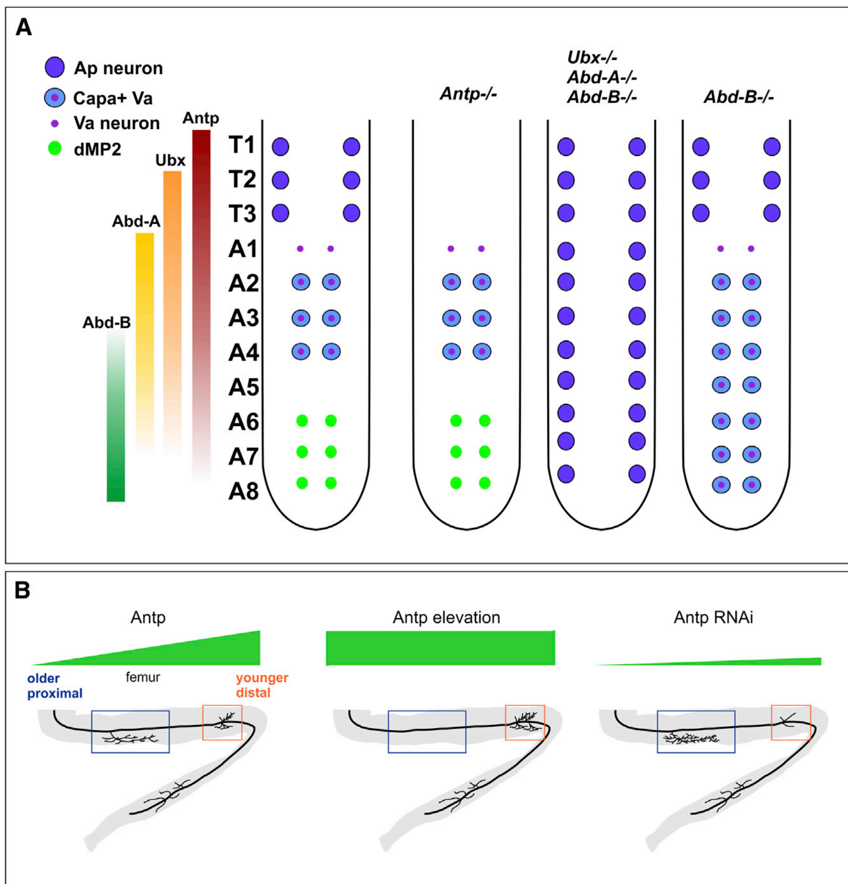


**Figure 6. *Hox* Genes Contribute to Neuronal Identity in the CNS of *Drosophila***

(A) *Hox* genes are essential for the generation of several types of peptidergic neurons in the ventral nerve cord of the fly embryo. *Hox* expression is shown relative to thoracic (T1–T3) and abdominal (A1–A8) segments. Peptidergic neurons are identified by expression of Apterous (Ap) and Capability (Capa) as well as other markers in Va and dMP2 populations. The distribution of these neurons is affected in *Hox* mutant embryos.

(B) Graded Antp activities contribute to the innervation of leg muscles in the fly. Elevation or depletion of Antp affects the branching patterns of motor axons at proximal and distal regions of the femur.

The disorganization of motor columns and pools observed in *Hox* mutants suggests regulation of cell adhesion properties by Hox proteins. Expression of the cadherin family of adhesion molecules, which mediate cell body organization of spinal MNs, is lost in *Foxp1*−/− mice (Dasen et al., 2008; Demireva et al., 2011). In *Hoxa1* mutants, cadherin6 expression is lost in r4 to r6 in the hindbrain, likely pointing to a role in rhombomere segregation (Inoue et al., 1997). Collectively, these studies indicate that *Hox* genes have a significant role in shaping neuronal organization and synaptic specificity in the CNS, through regulating the expression of large families of guidance and cell adhesion molecules.

## *Hox* Genes and the Diversity of Nervous Systems

The reliance on a Hox-based program for neuronal diversification presumably has allowed for a certain degree of flexibility in the ability of motor and sensory systems to adapt, as best evident in the variations in complexity and organization of vertebrate motor neurons (Fetcho, 1987, 1992). The prominent role of *Hox* genes in neuronal specification in vertebrates raises the question of whether they define a conserved mechanism for generating neuronal diversity along the rostrocaudal axis. There is emerging evidence that in addition to their more global roles in segmental patterning, *Hox* genes have essential roles in determining the identity of neuronal subtypes in invertebrates.

## *Hox* Genes in the Development of the Fly Nervous System

Studies in the *Drosophila* CNS demonstrate that *Hox* genes determine both the segment-specific distribution and subtype identity of neural populations in the embryonic and larval CNS. In contrast to the vertebrate CNS, where progenitors typically give rise to a single or only a few distinct neuronal classes, *Drosophila* embryos use a lineage-based strategy to generate diversity. As neural stem cells (neuroblasts) asymmetrically divide, they give rise to several classes of neuronal types, the identity of which depends on a temporal cascade of transcription factors that are transiently expressed in neuroblast lineages (reviewed in Skeath and Thor, 2003). The identity and distribution of these neurons depends in part on how temporally acting transcription factors intersect with rostrocaudal patterning cues.

The integration of temporal and rostrocaudal programs has been best studied in the specification of neuropeptide-producing cells generated in abdominal and thoracic segments of the fly embryo (Figure 6A). For example, the three thoracic segments (T1–T3) of the ventral nerve cord produce peptidergic neurons defined by the expression of the transcription factor Apterous. Generation of Apterous+ neurons relies on Hox activity, as these neurons are lost in mutants for the *Hox* gene *Antp* (Karlsson et al., 2010). Conversely, misexpression of Antp in a mutant background lacking posterior *Hox* genes gives rise to Apterous neurons in all segments. The conversion requires coexpression of the "temporal" transcription factor Grainy head, underscoring the importance of integrating Hox activities with the temporal specification code.

Peptidergic neuron specification also depends on Hox-dependent programs that impact neuronal number, through termination of cell-cycle progression or ablation of neurons that have already been generated. The absence of Apterous neurons in abdominal segments is not due to a cell-fate specification program, but rather the fact that the three posterior *Hox* genes, *Ubx*, *Abd-A*, and *Abd-B*, terminate cell cycle in the progenitors

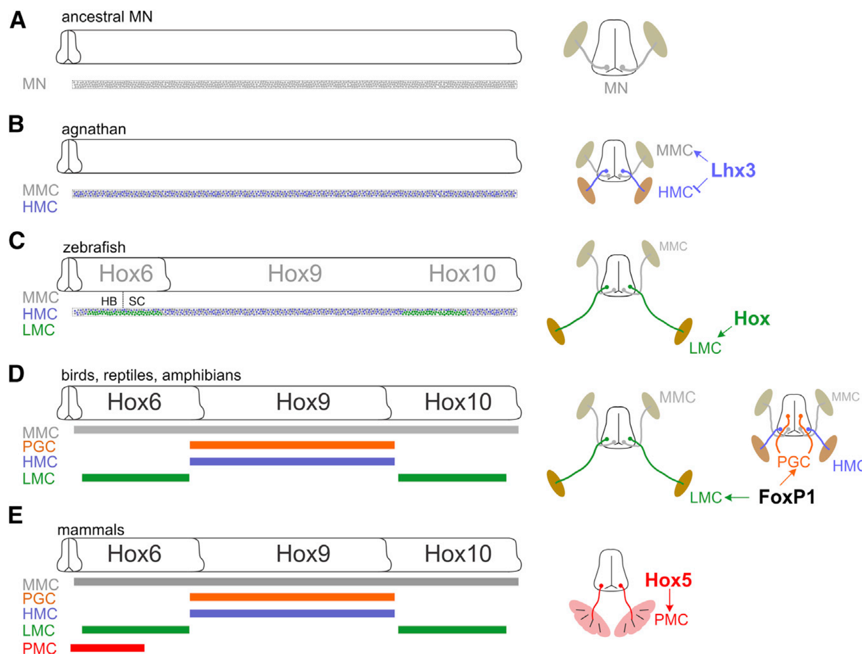


**Figure 7. A Model for the Emergence of Motor Neuron Diversity in Vertebrates**

(A) Ancestral MNs were characterized by the expression of a core set of transcription factors including Hb9, Isl1/2, and Lhx3, which are conserved in MNs of many invertebrates.

(B) Agnathan species lack paired appendages and likely contain MNs required for the innervation of dorsal and ventral muscles at all segments. For simplicity, we indicate these populations as "MMC" and "HMC" neurons, although they likely lack a columnar organization. The specification of ventrally projecting MNs may have required the exclusion of Lhx3, which defines dorsally projecting MMC neurons in tetrapods.

(C) Zebrafish pectoral fins are innervated by MN populations that appear to be generated in registry with *Hox6* and *Hox9* expression domains. MMC- and HMC-like MNs are not organized into columns.

(D) Tetrapod MNs display a columnar organization. Foxp1 probably played a key role in establishing both the columnar organization and multiple aspects of MN specification.

(E) In mammals, *Hox5* genes specify PMC MNs in a Foxp1-independent manner.

generating Apterous neurons. The *Hox* gene Abd-A contributes to the distribution of neurons within abdominal segments through induction of apoptosis (Bello et al., 2003), while Abd-B contributes to the specification of dMP2 neurons by preventing cell death (Miguel-Aliaga and Thor, 2004). Abd-B also promotes apoptosis of Va neurons to restrict their distribution to rostral abdominal segments (Suska et al., 2011). Thus, *Hox* genes can pattern the nervous system through mechanisms that are not solely dependent on activation of a set of cell-type-specific determinants.

*Hox* genes are also critical in the development of the neuromuscular network involved in locomotion. The embryonic nervous system of the fly generates motor circuits required for basic peristaltic movements that are distinct in specific rostrocaudal segments. *Hox* gene mutations have been shown to lead to transformation in these segment-specific patterns of motor activity (Dixit et al., 2008). Although flies and vertebrates use similar transcription factors to generate MNs as a class (Figure 7A) (Landgraf and Thor, 2006), there is little evidence that *Hox* genes contribute to MN subtype diversity in the fly embryonic nervous system. Thus, the locomotor defects observed in *Hox* mutant embryos probably reflect alterations in other neuronal or non-neuronal cell types.

During the larval stage of fly development, the nervous system generates additional neuronal populations to allow for the emergence of adult motor behaviors such as walking and flying. There is also evidence that *Hox* genes have important roles in the innervation of appendages such as the leg. Motor neurons innervating the leg are found in segments T1–T3, and within each segment nine neuroblast lineages give rise to ~50 MNs innervating 14 leg muscles (Baek and Mann, 2009; Truman et al., 2004). In contrast to vertebrates, the cell bodies of these MNs are not somatotopically organized, but rather their target specificity can be linked to birth order. One lineage, LinA, gives rise to 28 of the 50 leg MNs. Early-born neurons in LinA project their axons proximally in the leg, while later-born neurons project distally, suggesting that there is a temporal relationship between birth order and innervation pattern (Figure 6B).

Analyses of *Hox* gene mutants indicate an essential function in controlling the survival and identity of leg MNs (Figure 6B). Interestingly, Antp expression is graded in LinA MNs, with high levels found in late-born MNs and decreasing levels in early-born progeny (Baek et al., 2013). Genetic analysis indicates that Antp acts as a dose-dependent determinant of MN connectivity. Loss of Antp leads to a reduction in axonal branches at distal muscles, while elevation of Antp generates additional distal branches and fewer proximal. These results indicate that graded actions of Hox proteins may influence the connectivity of neurons with target cells.

### Evolution of Vertebrate Spinal MN Diversity

While studies in fly suggest conservation in the Hox-based program for neuronal diversification, it is yet to be determined how this program is varied in related species that exhibit distinct motor behaviors, such as walking, flying, and swimming. Comparisons of MN organization between modern species may shed light onto how the vertebrate motor system evolved (Figure 7).

The common ancestors of tetrapods and fish lacked limbs, raising the question of how the Hox-dependent program for appendicular muscle innervation emerged in vertebrates. Cephalochordates (e.g., amphioxus) and agnathan vertebrates (e.g., lamprey) exhibit colinear expression of *Hox* genes in the spinal cord (Schubert et al., 2006; Takio et al., 2007), although they lack limbs and other regionally restricted targets supplied by Hox-dependent MNs. Analyses in zebrafish provide some clues into the mechanisms through which the limb innervation program

probably emerged. MNs innervating pectoral fins localize to both the hindbrain and spinal cord (Ma et al., 2010), suggesting that the program for limb innervation originated through a Hox-dependent program that was initially linked to movement of the head (Figure 7C). Despite the altered position of fin-innervating MNs relative to tetrapods, they still retain an alignment with Hox expression domains in the CNS, such that the anterior boundary of Hox9 expression defines the caudal extent of pectoral fin-innervating populations (Prince et al., 1998). Thus, the boundary between limb-level Hox and Hox9 proteins appears to represent an early mechanism that segregated limb and non-limb MN identity. In contrast, MNs innervating the pelvic fin are not matched to Hox10 expression in the spinal cord, suggesting alternative Hox programs may be at work (Murata et al., 2010). Moreover, the discrete columnar grouping of MNs present in tetrapods has not been observed in fish species (Menelaou and McLean, 2012; Thorsen and Hale, 2007), indicating that if a Hox-dependent MN program is present, it is configured in such a way that only a subset of the programs present in tetrapods is activated.

Although forelimb and hindlimb LMC neurons are specified by distinct sets of *Hox* genes in tetrapods, they deploy nearly identical molecular programs initially; they express high levels of Foxp1 and Raldh2 and display similar LIM homeodomain codes. A primary target of *Hox* gene activity at limb levels is the induction of the transcription factor Foxp1 (Figure 7D), which is required for all subsequent aspects of LMC and PGC neuronal differentiation (Dasen et al., 2008). In *Foxp1* mutants, MNs revert to a molecular identity similar to the hypaxial motor column (HMC) neurons that normally reside in thoracic spinal cord. These observations suggest that HMC neurons represent the ancestral MN population from which Hox-dependent MNs emerged and was probably present at all rostrocaudal levels in more rudimentary vertebrates (Figure 7B).

Changes in Hox inputs to HMC-like MN populations appear to have continued to contribute to MN diversity. The phrenic motor column is unique to mammals, as the diaphragm muscle is not present in other tetrapod classes such as birds, amphibians, and lizards (Figures 7D and 7E). In the absence of *Hox5* genes in mice, PMC neurons are lost and animals perish due to respiratory failure (Philippidou et al., 2012). Interestingly, among brachially expressed Hox proteins, Hoxa5 shows a diminished ability for LMC induction (Lacombe et al., 2013), indicating that it may have evolved specifically to regulate PMC-specific gene targets. In the absence of *Foxp1*, Hox5$^{+}$ motor neurons at rostral cervical levels acquire molecular features and projection characteristics of PMC neurons. Thus, like LMC neurons, PMC neurons appear to have emerged from an HMC-like population that excluded LMC Hox determinants and acquired sensitivity to *Hox5* genes.

### *Hox* Genes as Substrates for Adaptability in Motor Systems

How might the Hox-based system for generating MN subtypes have been used as an adaptive strategy in the vertebrate lineage? A highly varied attribute in vertebrates is the distance between the forelimb and the hindlimb, which is defined by the number of thoracic segments and can be as few as four in amphibians or as many as 300 in some snake species. One question is how LMC neurons are generated in registry with the position of the limbs. Genetic analysis in mice has revealed that while multiple *Hox* genes are involved in forelimb and hindlimb LMC specification, the single *Hoxc9* gene specifies the position of thoracic MNs relative to the LMC (Jung et al., 2010). This strategy would in principle allow adaptability in the position of limb-innervating MN populations through changing the expression of a single transcription factor. Changes in the pattern of Hoxc9 expression among vertebrates could therefore contribute to the alignment of motor columns with their peripheral targets.

Another important question is how the specification of Hox-dependent LMC pools has been implemented to articulate limb muscles in diverse species. Both forelimb and hindlimb LMC populations deploy an initially similar molecular program but ultimately generate specific MN pools that are dedicated to innervating a single muscle in the limb (Dasen and Jessell, 2009). At this level, the Hox-dependent program becomes more selective, and specific Hox proteins act by deploying pool-restricted programs such as activation of intermediate transcription factors. This idea is exemplified by the roles of Hoxc6 and Hoxc8, which while both capable of imposing an LMC identity to thoracic MNs, have distinct functions in specifying motor pools (Lacombe et al., 2013; Tiret et al., 1998; Vermot et al., 2005). Thus, it appears that the Hox-dependent program of columnar differentiation uses a set of fairly permissive inputs early on (e.g., activation of *Foxp1*), but more specific activities emerge during motor pool specification. When one considers the diversity of locomotor strategies, for example, the use of the forelimb for walking versus wing muscles for flying, changes in *Hox* expression within LMC neurons could allow for alterations in the relative distribution of motor pools dedicated to innervating a muscle, without affecting their early columnar identity.

## *Hox* Genes and Neural Circuit Formation

While the connections between MNs and muscles are amongst the first to be established during development, it is the subsequent connectivity with premotor interneurons and sensory neurons that drives the basic wiring of circuits in the hindbrain and spinal cord. Formation of these neuronal networks is perturbed in the absence of *Hox* genes (Figure 8), suggesting a more global role in circuit assembly.

### *Respiratory Networks*

The neural networks that control respiratory rhythm generation reside in the brainstem (Figure 8A). Two distinct nuclei, the parafacial respiratory group (pFRG/RTN), derived from r3/r4 and the pre-Bötzinger complex (pre-BötC), derived from caudal rhombomeres (r6–r8), are the primary respiratory rhythm generators in the medulla, and mutations affecting their development lead to perinatal death due to respiratory failure (Bouvier et al., 2010; Rose et al., 2009). The pre-BötC appears to be the dominant respiratory pattern generator, with pFRG acting to entrain this rhythm and to initiate breathing during birth (Thoby-Brisson et al., 2009). Respiratory regions in the pons connect to and modulate medullary respiratory networks. These pontine respiratory structures are derived from rostral rhombomeres r1/r2. *Hoxa2* inactivation leads to an expansion of r1 at the expense of r2 and an increase in inspiratory amplitude, while breathing frequency remains unaffected, consistent with a role of pontine

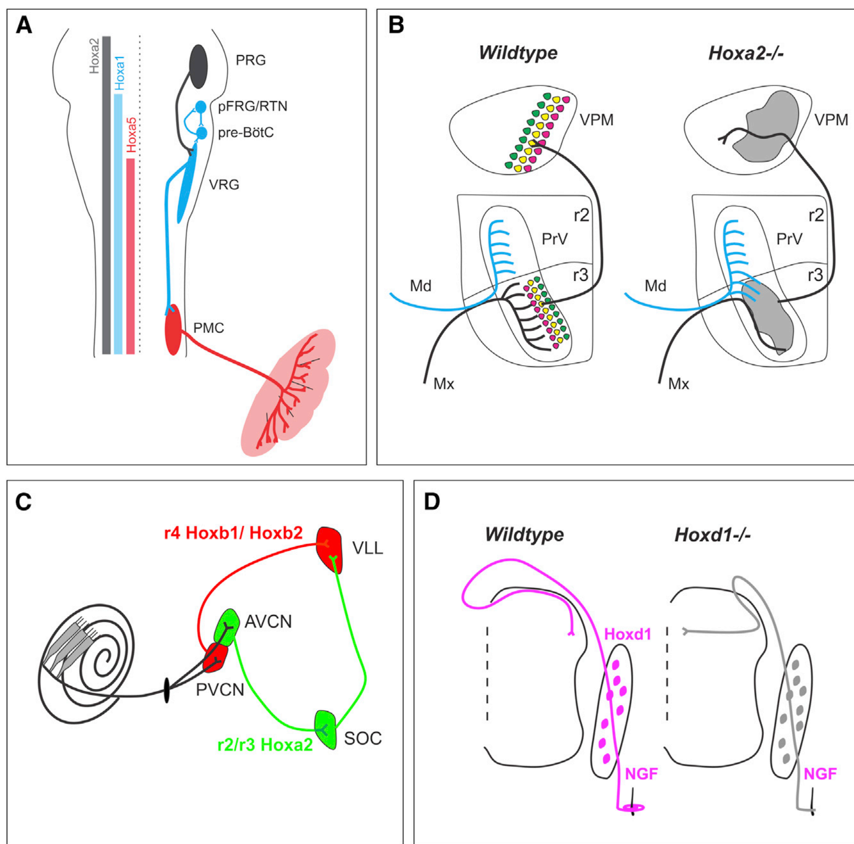


**Figure 8. *Hox* Genes in the Assembly of Neural Circuits**

(A) Multiple *Hox* genes mediate the assembly of respiratory networks in the hindbrain and spinal cord. The pontine respiratory group (PRG) relies on *Hoxa2* expression, while *Hoxa1* is involved in the development of the rhythmogenic parafacial respiratory group (pFRG) and *Hox5* genes control phrenic MN identity.

(B) *Hoxa2* is required for topographic projections from the trigeminal principal sensory nucleus (PrV) to the ventral posterior medial (VPM) nucleus of the thalamus. In the absence of *Hoxa2*, the maxillary (Mx) branch of the trigeminal nerve fails to arborize at the r3-derived domain of the PrV, which is ectopically targeted by the mandibular (Md) branch that normally innervates the r2-derived PrV. Projections from the r3-derived PrV are also mistargeted in the VPM and both the PrV and VPM lose their topographic organization.

(C) In the auditory system, r4-derived structures (red), such as the posteroventral cochlear nucleus (PVCN) and the ventral lateral lemniscus (VLL), form circuits that mediate sound perception, while r2/r3- and r5-derived structures (green), such as the anteroventral cochlear nucleus (AVCN) and the superior olivary complex (SOC), are primarily involved in circuits encoding sound localization. Mutations in *Hoxb1/Hoxb2* affect the sound perception pathway, while *Hoxa2* is involved in the wiring of the sound-localizing circuits. SOC is shown as one structure for simplicity.

(D) In mouse, but not chick, target-derived nerve growth factor (NGF) induces expression of *Hoxd1* in nociceptors. In the absence of *Hoxd1*, both peripheral and central connectivity of mouse nociceptors is altered to resemble chick nociceptor properties.

circuits in respiratory functions other than rhythm generation (Chatonnet et al., 2007). This phenotype is distinct from that of *Hoxa1* −/− mice, which die shortly after birth from breathing defects (Carpenter et al., 1993; Chisaka et al., 1992; Lufkin et al., 1991; Mark et al., 1993). The *Hoxa1* mutation does not affect the development of the caudally derived pre-BötC, but these mice show a hypoplasia in the r4 region where the pFRG is located, uncovering a role for this nucleus in preventing apneas at birth. Further analysis of *Hoxa1*−/− mice revealed the integration of ectopic r3/r4-derived neurons into a functional rhythm generating network, demonstrating rewiring of neuronal circuits upon alterations in *Hox* gene expression (del Toro et al., 2001). *Hox5* paralog genes control the development of the respiratory output phrenic MNs in the spinal cord (Philippidou et al., 2012), underscoring the importance of multiple *Hox* inputs in the wiring of respiratory circuits.

### *Formation of the Somatosensory Map*

The transmission of sensory information from the periphery to cortical areas relies on high-fidelity relay of sensory inputs to nuclei in the brainstem and thalamus. In the trigeminal pathway, sensory ganglia send projections to various facial areas and project centrally to distinct nuclei in the brainstem, such as the rostral principal (PrV) nucleus (Figure 8B). These nuclei in turn target nuclei in the thalamus, which connect to specific areas of the somatosensory cortex devoted to the representation of facial structures. There is a strict topographic organization of neuronal connections, such that each point in the periphery is mapped to a distinct area of the brainstem, thalamus, and cortex. *Hoxa2* has been shown to be a critical determinant in the formation of topographic somatosensory maps and acts at distinct phases to control multiple aspects of circuit connectivity (Oury et al., 2006). In the absence of *Hoxa2*, trigeminal sensory afferents inappropriately project to the cerebellum instead of terminating at the PrV nucleus. Late removal of *Hoxa2* retains correct trigeminal pathfinding to the PrV, but the maxillary branch of the ganglion, carrying information from the whiskers, upper jaw, and lip, fails to arborize. Examination of *Hoxa2*−/− mice at postnatal stages revealed that the topographic representation of inputs is eroded both in the PrV brainstem nucleus and in the thalamus and that PrV axons are mistargeted to other thalamic regions. The requirement for *Hoxa2* at various stages of trigeminal circuit formation exemplifies the diverse temporal and spatial roles of *Hox* genes in CNS development.

### *Auditory Circuits*

An example of how multiple *Hox* genes can contribute to circuit formation comes from a recent study delineating the roles of *Hoxb1*, *Hoxb2*, and *Hoxa2* in the assembly of auditory circuits (Figure 8C) (Di Bonito et al., 2013). Temporally controlled removal of these genes in the hindbrain bypassed early patterning defects and revealed novel roles in the specification and connectivity of auditory nuclei. While interpretation of *Hox* mutant phenotypes is confounded by the transcriptional interactions between these genes, the study sheds light on the temporally distinct steps of auditory circuit development. Afferent sensory

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fibers innervating inner and outer hair cells of the cochlea relay information to nuclei in the brainstem, which encompass the anteroventral (AVCN), posteroventral (PVCN), and dorsal (DCN) nuclei. Sensory information required for sound perception is transmitted from the cochlear nuclei through the lateral lemniscus complex and eventually reaches the auditory cortex through relay stations in the inferior colliculus of the midbrain and the medial geniculate nucleus of the thalamus. A parallel pathway emerging from cochlear nuclei and passing through nuclei of the superior olivary complex encodes sound localization.

The contribution of *Hox* paralogs to distinct circuit components aligns with their rhombomeric origin, with *Hoxb1/b2* determining r4-derived structures and *Hoxa2* being critical in r2/r3 structures. The PVCN is derived primarily from r4, while the AVCN is derived from r2/r3. In the absence of *Hoxb1* or *Hoxb2*, r4-derived structures acquire an r2/r3-like identity, leading to abnormal specification and axonal targeting of PVCN neurons to AVCN targets. AVCN neurons normally project to a nucleus in the superior olive that is important for sound localization and deletion of *Hoxa2* leads to defects in AVCN axon guidance, through downregulation of the guidance receptor *Rig1/Robo3*. The ventral lateral lemniscus, a target of PVCN neurons, is primarily derived from r4 and it is almost completely absent in *Hoxb1* mutants. *Hoxb1* and *Hoxb2* are also required for the correct specification of olivocochlear motor neurons innervating cochlear hair cells. Absence of motor innervation in *Hoxb1* and *Hoxb2* mutant mice results in abnormal morphology and loss of cochlear hair cells, leading to an increase in auditory threshold and a hearing impairment in these mice in adulthood. The data collectively point to a role for *Hoxb1/b2* and r4-derived structures in circuits controlling sound perception and amplification while *Hoxa2* primarily determines specification and connectivity in an r2/r3-dependent sound localization auditory circuit. Auditory circuits are a prime example of multiple *Hox* contribution in the assembly of a neuronal network.

### *Locomotor Circuit Assembly*

The execution of coordinated movement in mammals is a complex behavior that relies on the appropriate formation of multiple neuronal networks throughout the CNS. Two key components of this behavior are the relay of proprioceptive information to MNs in the spinal cord and the transmission of cortical motor and sensory input via the brainstem to the cerebellum. Both of these processes have recently been linked to Hox function, as changes in Hox activity or expression lead to a rewiring of these networks. In LMC neurons, a major output of Hox activity relies on the high expression of the Hox cofactor Foxp1. In global *Foxp1*−/− mice, multiple aspects of LMC development are affected and mice die at midembryonic stages due to heart failure (Wang et al., 2004). Conditional inactivation of *Foxp1* in MNs reveals additional defects in these mice that manifest at later stages. In the formation of motor-sensory circuits, Ia proprioceptive afferents centrally project to MNs innervating the same muscle. In the absence of Hox activity from MNs, this sensory-motor connectivity is perturbed, resulting in severe motor discoordination (Sürmeli et al., 2011). In the hindbrain, perturbing Hox5 paralog expression by eliminating the histone methyltransferase *Ezh2* leads to abnormal migration and ectopic formation of precerebellar pontine nuclei (Di Meglio et al., 2013). An additional contribution of *Hox* genes to movement control can be seen by their contribution to the vestibular system. *Hoxb1* activity is required for specifying the lateral vestibular nucleus (LVN), originating in r4, which modulates postural adjustments to movements (Chen et al., 2012).

### *Nociception*

An unexpected role for a *Hox* gene, unrelated to the specification of rostrocaudal identity, emerged from the analysis of genes induced by nerve growth factor (NGF) in nociceptors of dorsal root ganglia (DRG) (Guo et al., 2011). *Hoxd1* is the only *Hox1* paralog that is not expressed in the hindbrain and shows an atypical expression pattern in DRGs that is continuous along the rostrocaudal axis. *Hoxd1* is robustly induced by NGF in mouse, but not chick, and controls mammalian-specific features of nociceptor development such as peripheral target innervation and central axonal projections (Figure 8D). *Hoxd1*−/− mice exhibit behavioral defects consistent with decreased pain sensitivity and central projection patterns of nociceptors revert to the corresponding avian pattern. Remarkably, ectopic Hoxd1 expression in chick nociceptors is sufficient for the acquirement of murine nociceptor features, indicating a critical role for Hoxd1 in the evolution of nociceptive circuits.

## Conclusions

The establishment of neural circuits in the hindbrain and spinal cord relies on the coordination of many events that play out over the course of development, including neuronal differentiation, migration, axonal guidance, and synaptogenesis. Each of these steps depends on programs that can be genetically encoded and/or driven by neuronal activity. *Hox* genes appear to predominate in hardwired aspects of circuit connectivity as best evident by their multifaceted roles in MNs. However, it is important to note that MN differentiation and connectivity also depend on a number of apparently Hox-independent pathways, including progenitor subtype specification (Sabharwal et al., 2011) and activity-dependent pathfinding and gene regulatory programs (Hanson and Landmesser, 2004, 2006). Although the mechanisms through which *Hox* genes contribute to synaptic specificity within neural circuits are not fully resolved, some general principles have emerged from analysis of their roles in different contexts.

The coordinate expression of the same *Hox* gene in groups of neurons and their targets provides an attractive model for driving synaptic specificity during the assembly of neuronal circuits. This idea is supported by studies in the hindbrain demonstrating a requirement for *Hoxb1* both in facial MNs and tissues of the periphery for proper connectivity. Facial MNs generated in r4 innervate second branchial arch targets derived from neural crest cells that also originate from r4. Tissue-specific deletion of *Hoxb1* from neural crest cells results in axonal guidance defects and death of facial MNs, confirming a role for *Hoxb1* in target tissues (Arenkiel et al., 2004). A Hox-based matching system may resolve the question of synaptic specificity in circuits confined to specific rostrocaudal segments. For example, spinal interneurons display similar Hox expression profiles as MNs at the same rostrocaudal level (Dasen et al., 2005), and it is plausible that coordinated Hox activity contributes to their

connectivity. On the other hand, long-range projections such as those of spinocerebellar tract neurons, if Hox dependent, would appear to be governed by a different set of principles, as Hox expression is distinct between these neurons and their supraspinal targets.

An additional mechanism that could contribute to circuit formation is the control of neuronal migration and settling as a function of Hox activity. This idea is exemplified by sensory-motor connectivity in the spinal cord, where a scrambling of dorsoventral MN cell body position resulting from the inactivation of Hox-dependent programs leads to defects in synaptic specificity (Sürmeli et al., 2011). This phenotype appears to arise primarily from alterations in cell body position, as MNs receive proprioceptive inputs appropriate for their dorsoventral coordinates. However, in some cases, motor or sensory nuclei appear to receive appropriate input despite changes in their position. In *Pea3−/−* mice, for example, MNs innervating the triceps muscle show an altered position in the spinal cord but receive appropriate sensory input (Vrieseling and Arber, 2006). In *Ezh2−/−* mice, ectopic pontine nuclei also receive appropriate input from the cortex despite their aberrant position (Di Meglio et al., 2013). It is therefore likely that a combination of both positional and molecular cues defined by *Hox* genes orchestrate the specificity of connections during neural circuit formation.

Studies of *Hox* genes in CNS development have provided basic insights into the strategies through which a highly related group of transcription factors determines neuronal subtype identity. While there is compelling evidence that *Hox* genes play key roles in defining the identity, organization, and peripheral connectivity of motor neuron subtypes, and their target effectors are beginning to be defined, the contribution of *Hox* genes to synaptic specificity in neural circuits within the CNS remains to be resolved. As methodologies for tracing synaptic connectivity are improved, in conjunction with the ability to selectively deplete *Hox* genes from specific neuronal classes, the system is poised for a new set of discoveries that will undoubtedly reveal new and exciting roles for *Hox* genes in CNS development and function.

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