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Hoxc10 and Hoxd10 regulate mouse columnar, divisional and motor pool identity of lumbar motoneurons

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A central question in neural development is how the broad diversity of neurons is generated in the vertebrate CNS. We have investigated the function of *Hoxc10* and *Hoxd10* in mouse lumbar motoneuron development. We show that *Hoxc10* and *Hoxd10* are initially expressed in most newly generated lumbar motoneurons, but subsequently become restricted to the lateral division of the lateral motor column (lLMC). Disruption of *Hoxc10* and *Hoxd10* caused severe hindlimb locomotor defects. Motoneurons in rostral lumbar segments were found to adopt the phenotype of thoracic motoneurons. More caudally the lLMC and dorsal-projecting axons were missing, yet most hindlimb muscles were innervated. The loss of the lLMC was not due to decreased production of motoneuron precursors or increased apoptosis. Instead, presumptive lLMC neurons failed to migrate to their normal position, and did not differentiate into other motoneurons or interneurons. Together, these results show that *Hoxc10* and *Hoxd10* play key roles in establishing lumbar motoneuron columnar, divisional and motor pool identity.

KEY WORDS: *Hoxc10*, *Hoxd10*, Motoneuron specification, Spinal cord, Mouse

INTRODUCTION

A key question in neural development is how a seemingly uniform population of progenitor cells gives rise to the enormous diversity of neurons in the vertebrate CNS. One system that has been central to addressing this issue is the spinal cord motoneurons. Mature spinal cord contains a wide diversity of motoneuron populations that can be distinguished by their positions in the spinal cord, their peripheral targets, and the constellation of transcription factors and surface molecules they express (Jessell, 2000; Price et al., 2002), yet all are derived from a common progenitor pool.

Considerable progress has been made in defining the mechanisms that control specification and differentiation of spinal cord motoneurons. Early in development the spinal cord becomes patterned along both the dorsoventral and rostrocaudal axis, with motoneuron generation occurring in a restricted ventral domain (Jessell, 2000). Once generated, motoneurons become highly organized into lateral and medial motor columns (LMC and MMC, respectively), subdivisions (lateral and medial) of the columns, and motor pools (Fig. 1A), each with characteristic peripheral targets and a unique position in spinal cord (reviewed by Landmesser, 2001). Research carried out over the last decade, primarily at brachial levels, has implicated a regulatory network of Hox genes in establishing the columnar, divisional, and pool specification of motoneurons (Dasen et al., 2003; Dasen et al., 2005; Vermot et al., 2005). Much less is known about motoneuron specification and diversification at lumbar levels.

Hox10 genes are expressed in lumbar spinal cord in both chick (Lance-Jones et al., 2001) and mouse (Choe et al., 2006). In chick, ectopic expression of *Hoxd10* induces thoracic motoneurons to express markers characteristic of lateral LMC (lLMC) neurons and innervate limb muscles (Shah et al., 2004). Conversely, targeted disruption of *Hoxa10* and/or *Hoxd10* has been reported to perturb locomotor behavior, vertebral column segmentation, and peripheral nerve projections in a manner that suggests one or more lumbar segments have been transformed into thoracic segments (Carpenter et al., 1997; Lin and Carpenter, 2003; Rijli et al., 1995; Tarchini et al., 2005; Wahba et al., 2001). These results indicated a role for *Hoxa10* and/or *Hoxd10* in the generation of lumbar LMC motoneurons, but provide little insight into the cellular, developmental, or molecular mechanisms regulated by Hox10 genes. With these earlier studies as background, we disrupted both *Hoxc10* (whose function has not been previously analyzed in mice) and *Hoxd10*, and examined the expression and function of these Hox genes during development and differentiation of motoneurons, nerve innervation and muscle morphogenesis. We show that *Hoxc10* and *Hoxd10* are expressed in the right time and place to function in motoneuron patterning. In the absence of *Hoxc10* and *Hoxd10* function, motoneurons in rostral lumbar segments fail to establish an LMC and instead differentiate as thoracic neurons. Surprisingly, in more caudal segments the LMC consists almost entirely of medial LMC (mLMC) neurons with few, if any, motoneurons differentiating into lLMC neurons. Since nearly all thigh muscles become innervated by mLMC neurons from a reduced number of spinal segments, motor pools are also clearly disrupted in mutant animals. Together, our results show that *Hoxc10* and *Hoxd10* play major roles in specifying the columnar, divisional and motor pool identities of lumbar motoneurons. In addition, mutations in these genes have minor, but consistent, effects on hindlimb muscle development.

MATERIALS AND METHODS

Generation of *Hoxc10*$^{-/-}$/*Hoxd10*$^{-/-}$ mutant mice

The *Hoxa10* allele has been reported previously (Wahba et al., 2001). A *Hoxc10*^{RFP} knockout allele was generated by replacing the first exon with the red fluorescent protein (RFP) gene and a *Hoxd10*^{hrGFP} knockout allele was generated by replacing the first exon with the humanized Renilla green fluorescent protein (hrGFP) gene (Fig. 2A). To generate the *Hoxc10*^{RFP} knockout allele, an 8.9 kb *Xho*I-*Sal*I genomic fragment spanning the two *Hoxc10* exons was used to construct the targeting vector. A reporter *neo* cassette containing the RFP, DsRed2 (Clontech) cDNA and a self-excision neomycin resistance gene (*neo*), was used to replace the first exon of *Hoxc10*, the first five amino acids of Hoxc10 being left intact and in-frame

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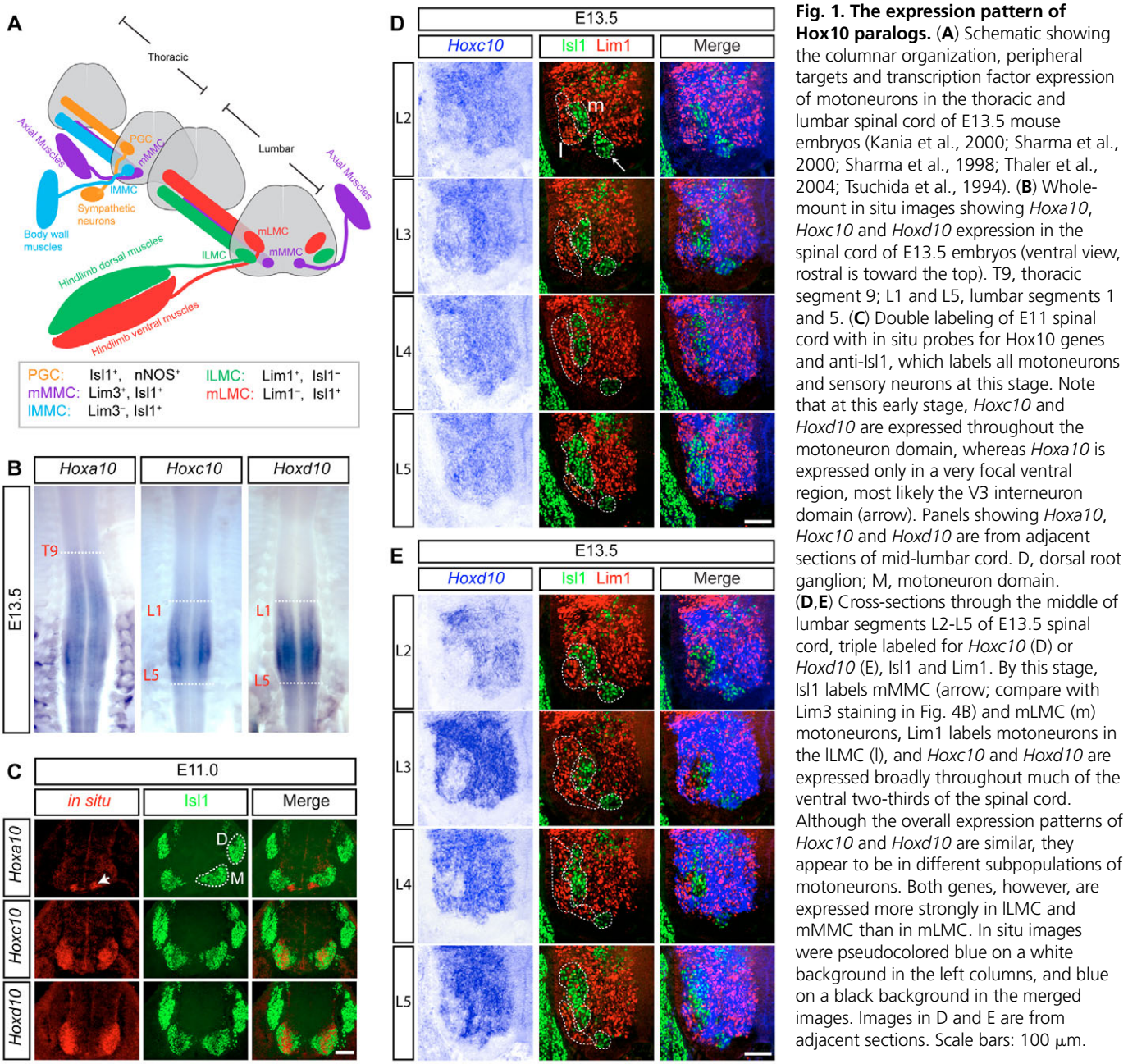


Fig. 1. The expression pattern of Hox10 paralogs. (**A**) Schematic showing the columnar organization, peripheral targets and transcription factor expression of motoneurons in the thoracic and lumbar spinal cord of E13.5 mouse embryos (Kania et al., 2000; Sharma et al., 2000; Sharma et al., 1998; Thaler et al., 2004; Tsuchida et al., 1994). (**B**) Whole-mount in situ images showing *Hoxa10*, *Hoxc10* and *Hoxd10* expression in the spinal cord of E13.5 embryos (ventral view, rostral is toward the top). T9, thoracic segment 9; L1 and L5, lumbar segments 1 and 5. (**C**) Double labeling of E11 spinal cord with in situ probes for Hox10 genes and anti-Isl1, which labels all motoneurons and sensory neurons at this stage. Note that at this early stage, *Hoxc10* and *Hoxd10* are expressed throughout the motoneuron domain, whereas *Hoxa10* is expressed only in a very focal ventral region, most likely the V3 interneuron domain (arrow). Panels showing *Hoxa10*, *Hoxc10* and *Hoxd10* are from adjacent sections of mid-lumbar cord. D, dorsal root ganglion; M, motoneuron domain. (**D**,**E**) Cross-sections through the middle of lumbar segments L2-L5 of E13.5 spinal cord, triple labeled for *Hoxc10* (D) or *Hoxd10* (E), Isl1 and Lim1. By this stage, Isl1 labels mMMC (arrow; compare with Lim3 staining in Fig. 4B) and mLMC (m) motoneurons, Lim1 labels motoneurons in the lLMC (l), and *Hoxc10* and *Hoxd10* are expressed broadly throughout much of the ventral two-thirds of the spinal cord. Although the overall expression patterns of *Hoxc10* and *Hoxd10* are similar, they appear to be in different subpopulations of motoneurons. Both genes, however, are expressed more strongly in lLMC and mMMC than in mLMC. In situ images were pseudocolored blue on a white background in the left columns, and blue on a black background in the merged images. Images in D and E are from adjacent sections. Scale bars: 100 μm.

with RFP. To construct the *Hoxd10* targeting vector, a 9.3 kb *Eco*RI-*Spe*I genomic fragment spanning the two *Hoxd10* exons was used. A reporter-*neo* cassette containing an hrGFP (Stratagene) cDNA and a self-excision *neo* was used to replace the first exon of *Hoxd10*, the first three amino acids of Hoxd10 being left intact and in-frame with hrGFP. In addition, the thymidine kinase gene (*Tk1*) was included in the final targeting vectors of *Hoxc10* and *Hoxd10*. To allow removal of the *neo* selection cassette subsequent to its use for isolation of targeted ES cell lines, the selectable gene *neo*, is embedded in a Cre/*lox*P-based self-excision cassette, in which Cre expression is mediated by a promoter, ACE, that is not expressed in ES cells, but is expressed in the male germline of mouse chimeras derived from these ES cells during spermatogenesis (Bunting et al., 1999).

The linearized targeting vectors were used for gene targeting in R1-45 embryonic stem (ES) cells. For *Hoxc10*, four out of 72 clones were confirmed by Southern blot to have undergone correct homologous recombination. For *Hoxd10*, two out of 144 clones were confirmed positive. One positive ES cell clone for *Hoxc10* or *Hoxd10* was injected into blastocysts to produce male chimeras, which were further backcrossed to C57BL/6j females. Among brown-colored offspring, heterozygotes were obtained for both Hox genes. The following PCR primers were used for genotyping: $Hoxc10^{RFP}$ (primer 1: 5′-AGATGTCAGCTCCTCCGCTGTAGT-3′; primer 2: 5′-GTCACCTTCAGCTTCACGGTGTT-3′; primer 3: 5′-AACAGGTTGTTCCAGGCGGTAG-3′; the mutant band is 248 bp and the wild-type band is 330 bp) and $Hoxd10^{hrGFP}$ (primer 1: 5′-CAAATCTCATTGGCTTGGTTGTCA-3′; primer 2: 5′-CTCCAGGTTCACCTTGAAGCTCAT-3′; primer 3: 5′-AAGATCTGTTCGGGTCTGTCCAAC-3′; the mutant band is 234 bp and the wild-type band is 385 bp). PCR conditions were: 94°C 30 seconds, 59.5°C 30 seconds and 72°C 30 seconds, for 31-35 cycles.

Immunohistochemistry and in situ hybridization

Immunostaining and in situ hybridization were performed as previously described (Boulet and Capecchi, 1996; Huber et al., 2005; Wang and Scott, 2007). The following primary antibodies were used: mouse anti-

Isl1 [1:50, 39.4D5, Developmental Studies Hybridoma Bank (DSHB)]; rabbit anti-Isl1 (1:2000, provided by Dr S. Pfaff, Salk Institute, San Diego, CA); rabbit anti-Hb9 (also known as Mnx1 – Mouse Genome Informatics; 1:8000, provided by Dr S. Pfaff); rabbit anti-Lim3 (also known as Lhx3 – Mouse Genome Informatics; 1:2000, provided by Dr S. Pfaff); rabbit anti-Lim1 (also known as Lhx1 – Mouse Genome Informatics; 1:20,000, provided by Dr T. M. Jessell, Columbia University, New York); rabbit anti-nNOS (1:5000, ImmunoStar, Hudson, WI); rabbit anti-Olig2 (1:20,000, provided by Dr J. Alberta, Harvard University, Boston, MA); sheep anti-Chx10 (also known as Vsx2 – Mouse Genome Informatics; 1:1000, Exalpha, Maynard, MA); mouse anti-myosin (1:4000, my32, Sigma); rabbit anti-MyoD (1:50, Santa Cruz); mouse anti-neurofilament 165 (1:50, 2H3, DSHB); mouse anti-βIII-tubulin (1:1000, Sigma); mouse anti-BrdU antibody (1:5, G3G4, DSHB). Species-specific Alexa Fluor 488- and Alexa Fluor 546-conjugated secondary antibodies (Invitrogen) were used at 1:1000. A *Raldh2* (also known as *Aldh1a2* – Mouse Genome Informatics) probe (provided by Dr Song Wang from our laboratory) was transcribed from a 603 bp cDNA fragment (1571-2173 bp; NM_009022). *Hoxa10* probe was transcribed from a 1043 bp cDNA fragment (1199-2241 bp; NM_008263). *ER81* (also known as *Etv1* – Mouse Genome Informatics) and *Pea3* (also known as *Etv4* – Mouse Genome Informatics) plasmids were provided by Dr S. Arber, University of Basel, Switzerland; the *Sema3E* plasmid was provided by Dr T. M. Jessell. Other template plasmids (*Hoxc10*, *Hoxd10*, *Hoxc9*, *Hoxd9*, *Hoxc11* and *Hoxc11*) were created in our laboratory (Hostikka and Capecchi, 1998).

To combine in situ hybridization and immunolabeling, sections were first processed for in situ hybridization with digoxigenin-labeled probes, visualized with NBT-BCIP, and then immunolabeled. In situ images were pseudocolored prior to combining with images of immunostaining.

Cell death in motoneurons was assessed by TUNEL (Cell Death Detection Kit; Roche) on Isl1- or Hb9-labeled sections. Mitotic cells were labeled with bromodeoxyuridine (BrdU; Sigma) injected intraperitoneally (50 μg/g body weight) into E9.5-E10.75 timed pregnant mice, and subsequently identified with motoneuron markers and anti-BrdU.

Anterograde and retrograde labeling

Axon projections from motoneurons in specific spinal cord levels were labeled with DiI (1,1′-dioctadecyl-3,3,3′3-tetramethylindocarbocyanine perchlorate; 2.5 mg/ml dimethylformamide; Molecular Probes, Eugene, OR) in paraformaldehyde-fixed embryos (Carpenter et al., 1993). Retrograde labeling of motor pools was achieved by injecting tetramethylrhodamine dextran [(3000 MW; Invitrogen) in Tris-buffered saline (TBS) with 10% lysophosphatidyl choline (Sigma)] into individual muscles (Vrieseling and Arber, 2006).

RESULTS

Hoxc10 and *Hoxd10* are expressed exclusively in the lumbar spinal cord

To gain insight into the function of Hox10 genes during embryonic development, we analyzed their expression patterns in lumbar spinal cord from E10.0-E16.5. Mouse spinal cord has six lumbar segments, with hindlimb muscles being innervated by LMC motoneurons in segments L1-L5 (Lance-Jones, 1982; Lin and Carpenter, 2003; Tarchini et al., 2005). All three Hox10 transcripts were first detected in lumbar spinal cord at E10.5, and the rostrocaudal expression domain of each did not change in any of the ages examined (data not shown). By E13.5 *Hoxc10* and *Hoxd10* were expressed most intensely in segments L2-L4, with lower levels of expression in L1 and segments caudal to L4 (Fig. 1B). Whereas *Hoxc10* and *Hoxd10* were expressed only in lumbar regions, *Hoxa10* expression extended from T10 to lumbar levels (Fig. 1B) (Choe et al., 2006).

Transverse serial sections confirmed the timing and rostrocaudal extent of expression of all three Hox10 transcripts (Fig. 1C-E). Furthermore, by staining these sections with antibodies to distinguish different motor columns (Fig. 1A), we were able to determine the cell types that expressed different Hox10 transcripts, and show that each transcript had a unique, highly dynamic pattern of expression. Postmitotic motoneurons, identified by Islet1 (Isl1) expression, first appeared in lumbar spinal cord around E10.0-E10.5 (data not shown), slightly later than reported for brachial spinal cord (Arber et al., 1999), and by E11 most, if not all, postmitotic motoneurons in segments L2-L4 expressed *Hoxc10* and *Hoxd10* (Fig. 1C). By contrast, *Hoxa10* was expressed in a very focal ventral domain, overlapping the

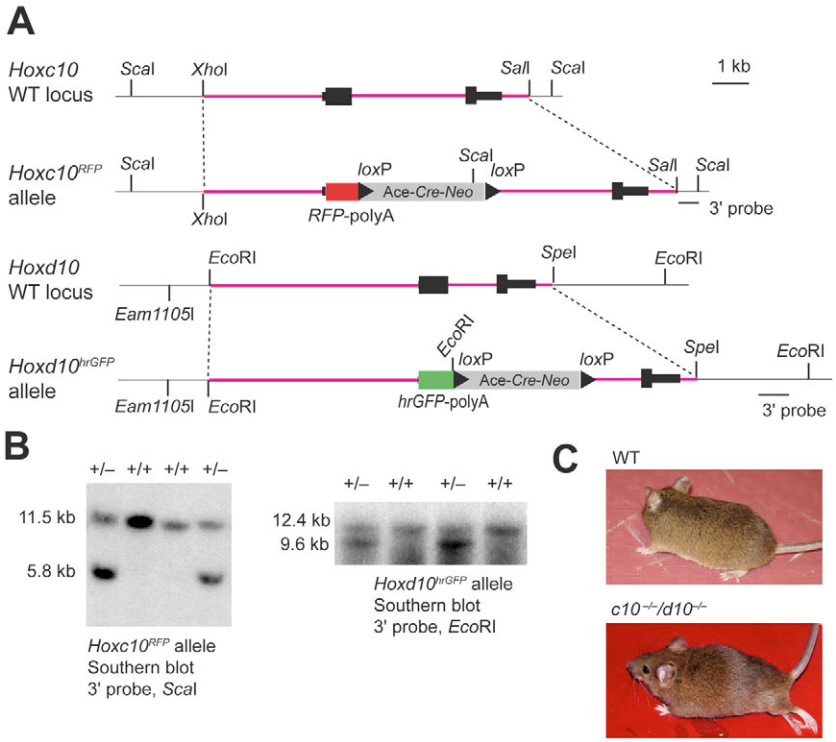


Fig. 2. Generation of *Hoxc10* and *Hoxd10* knockout alleles. (**A**) Schematic showing the wild-type (WT) genomic loci for *Hoxc10* and *Hoxd10*, as well as targeted alleles for *Hoxc10* and *Hoxd10*. (**B**) Southern blot analysis of DNA from ES cell clones for $Hoxc10^{RFP}$ and $Hoxd10^{hrGFP}$. For $Hoxc10^{RFP}$, *Sca*I digestion and a 3′ flanking probe were used. The WT band is 11.5 kb and the correctly targeted band is 5.8 kb. For $Hoxd10^{hrGFP}$, *Eco*RI digestion and a 3′ flanking probe were used. The corresponding WT band is 12.4 kb, and the mutant band is 9.6 kb. (**C**) A typical hindlimb posture for $Hoxc10^{-/-}/Hoxd10^{-/-}$ mutants and WT control.

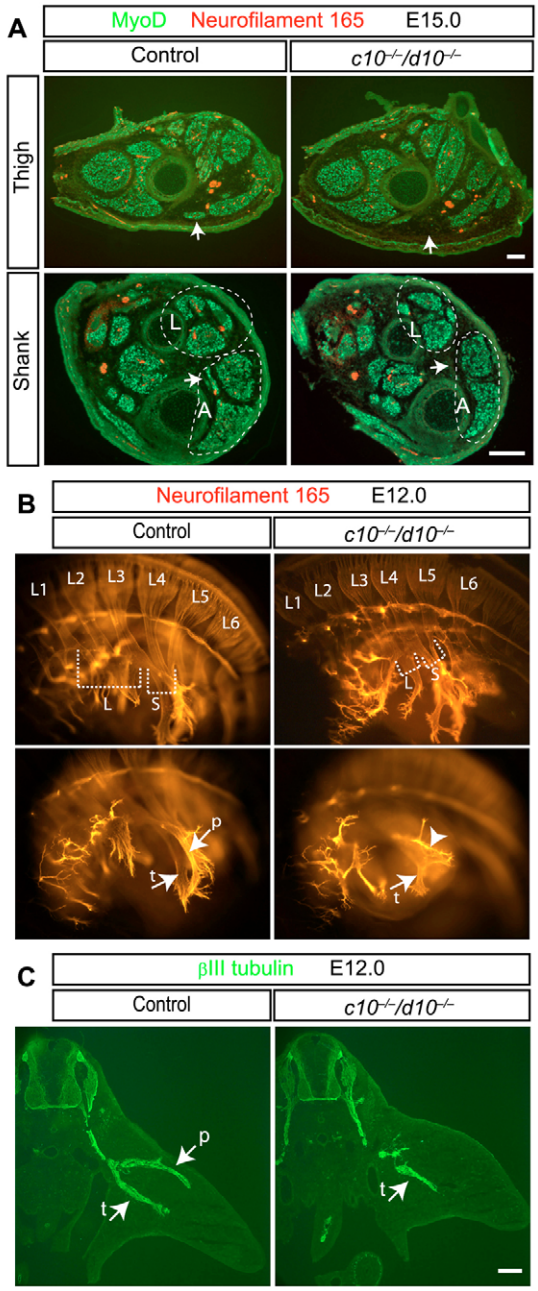


Fig. 3. Defects in muscle development, innervation and axon projections in *Hoxc10*$^{-/-}$/*Hoxd10*$^{-/-}$ double-mutant embryos. (**A**) Cross-sections through mid-thigh and mid-shank of embryos stained for muscle (MyoD) and nerve (neurofilament 165). Two muscles, the anterior head of the biceps in the thigh and the extensor hallucis longus in the shank (arrows) are missing in this *Hoxc10*$^{-/-}$/*Hoxd10*$^{-/-}$ mutant embryo. In the shank of these mutants, innervation is completely lost from muscles of the anterior group (A) and greatly reduced in the lateral group (L). (**B**) Whole-mount neurofilament staining of axons in the hindlimb (lateral view, rostral is to the left). Top panels show the contribution of spinal segments to the lumbar (L) and sacral (S) plexii. In control embryos, segments L1-L3 contribute to the lumbar plexus and L3-L5 to the sacral plexus. In double-mutant embryos, axons in the two plexii were derived from more-caudal segments; L3 and L4 contributed to the lumbar plexus and L4 and L5 to the sacral plexus. Bottom panels are higher magnification images showing that the dorsal peroneal nerve (p) of the sacral plexus is missing in *Hoxc10*$^{-/-}$/*Hoxd10*$^{-/-}$ mutant embryos, whereas the ventral tibial nerve (t) is still present. Arrowhead indicates a cutaneous nerve. (**C**) Cross-sections of lumbar spinal cord and hindlimb, through the sacral plexus, stained for β-III-tubulin. In control embryos, both the dorsal (peroneal, p) and ventral (tibial, t) branches were present, whereas in *Hoxc10*$^{-/-}$/*Hoxd10*$^{-/-}$ mutants only the ventral branch was detected. Scale bars: 200 μm.

characteristic position of the V3 interneuron domain (Fig. 1C) (Briscoe et al., 1999), and extending rostrally into thoracic spinal cord (data not shown).

At later stages, from E11.5 onward, *Hoxa10* is expressed in motoneurons (data not shown). Although expression of all three paralogs expanded to cells throughout much of the ventral two-thirds of the spinal cord, *Hoxc10* and *Hoxd10* transcripts are lost from many motoneurons with each becoming restricted to specific populations of motoneurons (Fig. 1D,E). For example, at E13.5, the stage when LMC and MMC neurons have segregated into distinct motor columns, both genes are weakly expressed in Isl1^{+} mMMC neurons in caudal L1 and throughout segment L2, but are almost undetectable in the LMC. At L3, although both *Hoxc10* and *Hoxd10* are expressed in Lim1^{+} lLMC motoneurons (in addition to the mMMC), they appear to be expressed in different subpopulations of motoneurons, most likely in different motor pools. At L4 and L5, whereas *Hoxc10* or *Hoxd10* are expressed throughout the lLMC and mMMC, a small number of Isl1^{+} mLMC neurons also express *Hoxc10* and *Hoxd10* (Fig. 1D,E).

The posterior expression limits of Hox10 genes are more easily defined in section in situ hybridizations than in whole-mount preparations. *Hoxc10* and *Hoxd10* expression in motoneurons extends only through L5 (data not shown). These genes are also expressed at extremely low levels in intermediolateral regions in more caudal spinal cord, which could account for the caudal expression of Hox10 genes observed in whole-mount preparations (Carpenter, 2002) (Fig. 1B). Taken together, our data show that *Hoxc10* and *Hoxd10* are expressed in motoneurons exclusively in lumbar spinal cord. The fact that both *Hoxc10* and *Hoxd10* are initially expressed in almost all newly generated motoneurons but later become restricted to subpopulations of motoneurons, primarily to lLMC and mMMC neurons, suggests they may play sequential roles in specifying or maintaining motoneuron identity at different stages. Conversely, the absence of *Hoxa10* from motoneurons at early stages suggests that this paralog may have relatively little influence on motoneuron specification. Moreover, because *Hoxc10* and *Hoxd10* have similar, although not identical, expression patterns in the developing spinal cord, they may share redundant functions in motoneuron development.

Generation of *Hoxc10* and *Hoxd10* double mutants

To examine the roles of Hox10 genes in the development of hindlimb motoneurons, we analyzed different combinations of Hox10 double and triple-mutant mice. The *Hoxa10* allele has been reported previously (Wahba et al., 2001). Here we describe mice carrying new alleles of *Hoxc10* and *Hoxd10* that lacked the *neo* cassette (Fig. 2), which we created because the presence of *neo* can affect the mouse phenotype by altering the expression of nearby genes (Greer and Capecchi, 2002; Manley et al., 2001). This is

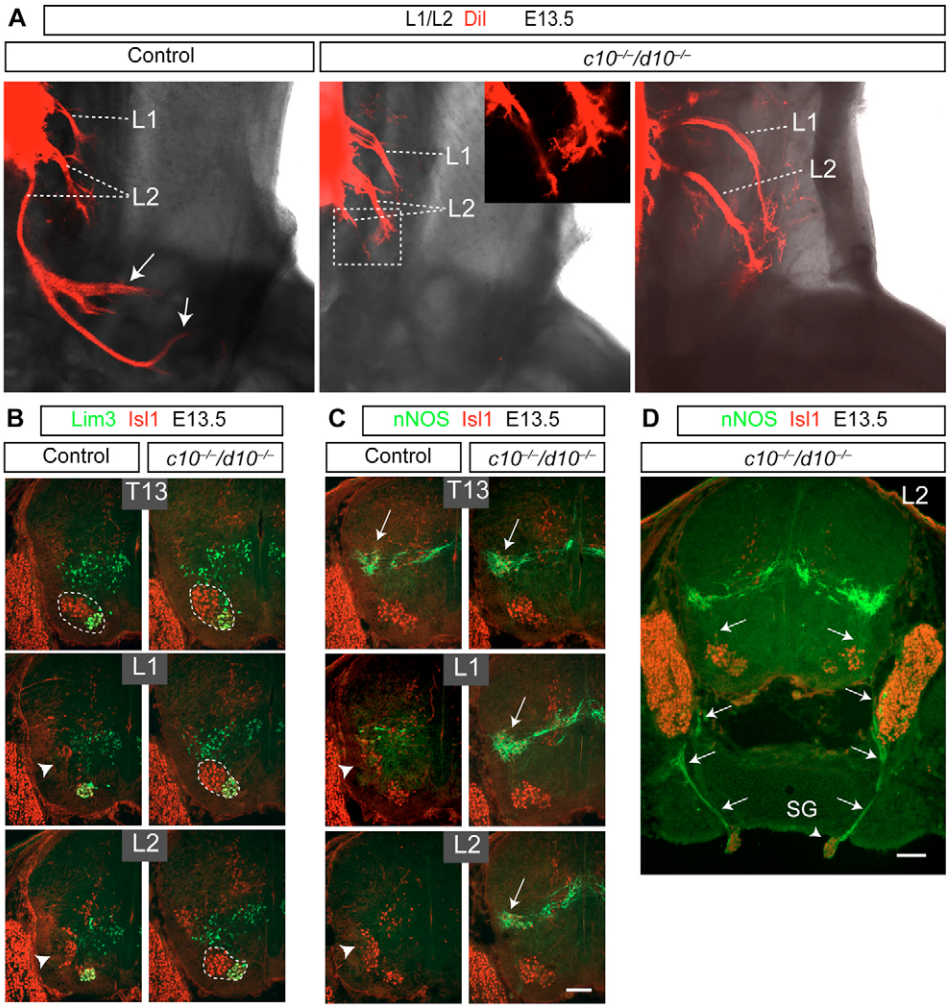


Fig. 4. Motoneurons in segments L1 and L2 assume identities of thoracic motoneurons in *Hoxc10*$^{-/-}$/*Hoxd10*$^{-/-}$ double-mutant embryos. (**A**) Whole-mount ventral view of DiI-labeled axons from lumbar segments L1 and L2. In the control embryo, axons from segment L2 project to the limb (arrows), but in both double-mutant embryos these axons project to the body wall. Inset shows boxed area with axon terminations of L2 at higher magnification. (**B**) Cross-sections through ventral spinal cord segments T13-L2 of E13.5 control and *Hoxc10*$^{-/-}$/*Hoxd10*$^{-/-}$ mutant embryos stained with anti-Lim3, which labels mMMC neurons, and with anti-Isl1, which labels the entire MMC and mLMC neurons. In control embryos, the pattern of labeled motoneurons differs significantly between thoracic and lumbar segments, whereas in *Hoxc10*$^{-/-}$/*Hoxd10*$^{-/-}$ mutants the thoracic pattern extends into lumbar cord. Dashed lines encircle the MMC. Arrowhead, mLMC neurons. (**C**) Cross-sections through ventral spinal cord segments T13-L2 of E13.5 control and *Hoxc10*$^{-/-}$/*Hoxd10*$^{-/-}$ mutant embryos stained with anti-nNOS (arrow) to label the PGC motor column in the intermediolateral spinal cord and with anti-Isl1 to label MMC and mLMC (arrowhead) motor columns in the ventral horn, as well as PGC motoneurons. In control embryos, nNOS is expressed in thoracic segments, with expression extending only to rostral L1. In *Hoxc10*$^{-/-}$/*Hoxd10*$^{-/-}$ mutant embryos, however, nNOS expression extended beyond segment L2 (arrow). (**D**) Cross-section through lumbar segment L2 of *Hoxc10*$^{-/-}$/*Hoxd10*$^{-/-}$ mutant embryo stained with anti-nNOS and anti-Isl1. Note that nNOS^{+} axons project to the sympathetic ganglia. Arrows point to axonal projection path. Arrowhead indicates sympathetic ganglion (SG). Scale bars: 100 μm for B-D.

particularly a problem with Hox genes, since the density of genes is high within this complex. Homozygous mutant mice were found to lack *Hoxc10* or *Hoxd10* transcripts when examined by in situ hybridization (data not shown), and thus appear to be null mutants. Both single and double-mutant animals were viable and had an apparently normal lifespan, although the double mutants were sterile. Double heterozygotes and single homozygous mutants did not have any obvious alteration in gait, and therefore heterozygotes were sometimes used with wild-type (WT) animals as controls in this study. The lack of an aberrant phenotype in double heterozygous and single homozygous mutants differs from the phenotypes of previously generated *Hoxd10* null mutants, which had obvious defects in locomotor behavior (Carpenter et al., 1997; Tarchini et al., 2005). The more normal behavior observed in our single mutants most likely results from the lack of the *neo* gene in our alleles. The lack of apparent locomotor phenotypes associated with mutations in either *Hoxc10* or *Hoxd10* alone also emphasizes the redundant functions of these Hox genes.

Animals carrying three mutant alleles had obvious locomotor defects, which varied in severity among different animals. *Hoxc10*$^{-/-}$/*Hoxd10*$^{-/-}$ double-mutant animals had even more severe defects in locomotion. Hindlimbs in these animals were held crossed in a rigid, extended position, and were not used to support body weight or for walking in an alternating right-left fashion. This phenotype was nearly invariant among *Hoxc10*$^{-/-}$/*Hoxd10*$^{-/-}$ mutant animals and was 100% penetrant (n>20; Fig. 2C).

We next compared *Hoxc10*$^{-/-}$/*Hoxd10*$^{-/-}$ double-mutant animals with *Hoxa10*$^{-/-}$/*Hoxc10*$^{-/-}$ and *Hoxa10*$^{-/-}$/*Hoxd10*$^{-/-}$ double mutants. Locomotor defects in *Hoxc10*$^{-/-}$/*Hoxd10*$^{-/-}$ mutants were significantly more severe than in the latter two groups. Surprisingly, locomotor defects in the Hox10 triple-mutant animals (n=3) seemed to be less severe than in *Hoxc10*$^{-/-}$/*Hoxd10*$^{-/-}$ double mutants, although the triple-mutant mice died around weaning, as a result of kidney defects. Thus, it appears that the loss of *Hoxa10* does not significantly contribute to defects in locomotor behavior, most likely because *Hoxa10* is not expressed within the motoneuron domain at early stages (Fig. 1C). Given the less prominent expression pattern of *Hoxa10* in the lumbar motor column, and the negligible additional contribution of the *Hoxa10* mutation to the *Hoxc10*$^{-/-}$/*Hoxd10*$^{-/-}$ mutant phenotype, we focused further analysis primarily on *Hoxc10*$^{-/-}$/*Hoxd10*$^{-/-}$ double-mutant animals.

Hindlimb muscle morphology and innervation patterns in *Hox10* mutants

Severe locomotion defects could result from either motoneuron projection errors and/or altered muscle patterning. Analysis of the overall pattern of muscle (Greene, 1935) and nerve innervation in cross-sections through the hindlimbs of E14.5-E15.0 mutant and WT embryos revealed that the anterior head of the biceps was missing from the thigh in four out of seven *Hoxc10*$^{-/-}$/*Hoxd10*$^{-/-}$ mutant embryos. In the shank, the extensor hallucis longus was missing from the anterior group in four out of four double-mutant embryos, and two more muscles were missing from the lateral group in three out of four double-mutant embryos (Fig. 3A and see Fig. S1 in the supplementary material). The loss of these muscles was confirmed by dissecting P0 hindlimb muscles stained with AP-conjugated anti-myosin (data not shown). To our knowledge, this is the first report of muscle defects associated with *Hox10* mutant animals.

Although a few muscles failed to form normally, this minor disruption in muscle patterning is unlikely to account for the very severe gait abnormalities observed in *Hoxc10*$^{-/-}$/*Hoxd10*$^{-/-}$ mutant animals. By contrast, nerve patterning was markedly abnormal in these double mutants. Axons were clearly detected in all muscles in cross-sections through the thigh of *Hoxc10*$^{-/-}$/*Hoxd10*$^{-/-}$ mutants. In the shank, however, muscles in both the anterior and lateral groups received no, or greatly reduced, innervation (Fig. 3A and see Fig. S1 in the supplementary material). In contrast to the *Hoxc10*$^{-/-}$/*Hoxd10*$^{-/-}$ mutants, there were no apparent muscle patterning or innervation defects in mice homozygous for the single, new *Hoxc10*$^{-/-}$ or *Hoxd10*$^{-/-}$ mutant alleles (see Fig. S1 in the supplementary material), consistent with the observation that these single-mutant mice show no obvious locomotor defects.

The lack of innervation to anterior and lateral shank muscles in the double mutants was confirmed in whole-mount embryos stained with neurofilament antibody at E12. Importantly, neurofilament

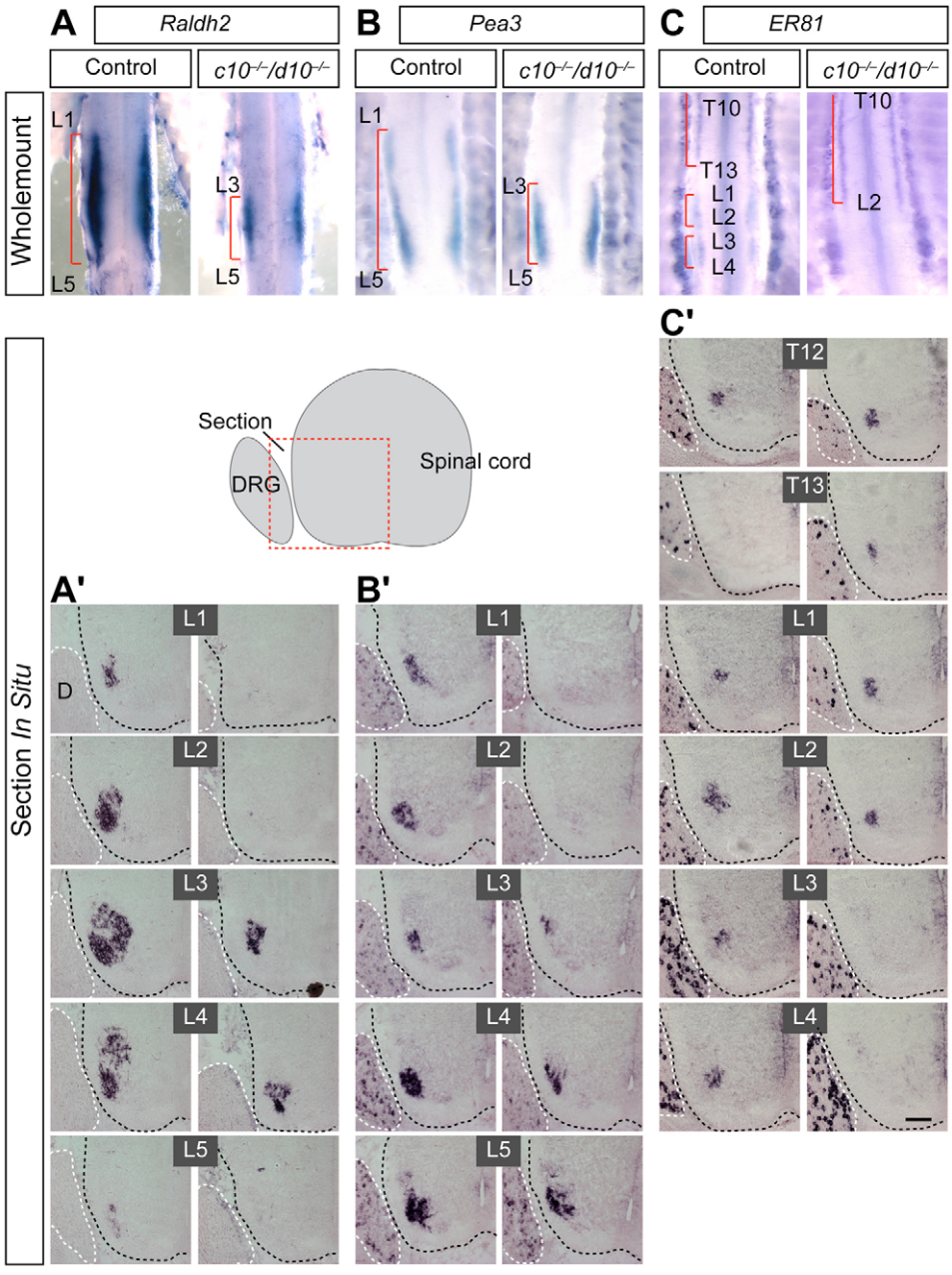


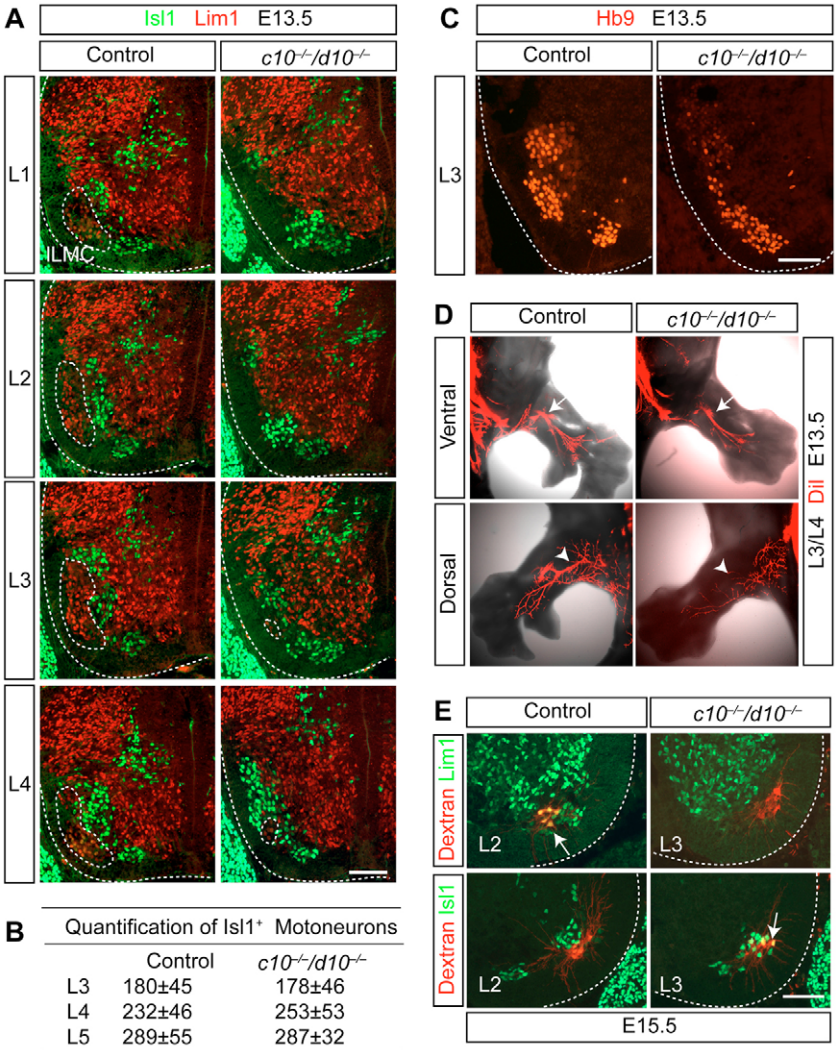
Fig. 5. Expression patterns of *Raldh2*, *Pea3* and *ER81* are altered in *Hoxc10*$^{-/-}$/*Hoxd10*$^{-/-}$ double-mutant embryos. (**A-C′**) In situ hybridization of *Raldh2*, *Pea3 and ER81* in (A-C) whole-mount spinal cord (ventral view, rostral is to the top) and (A′-C′) cross-sections through ventral spinal cord (outlined in black), as indicated in the drawing; lateral is to the left in each panel. D, dorsal root ganglion, outlined in white. Note that in *Hoxc10*$^{-/-}$/*Hoxd10*$^{-/-}$ mutant embryos, *Raldh2* and *Pea3* expression was restricted to segments L3-L5, being absent from segments L1 and L2. By contrast, *ER81* expression extends from thoracic segments into segments L1 and L2, but is lost from segments L3 and L4 in mutants. Scale bar: 100 μm for A′-C′.

staining also revealed striking abnormalities in the contribution of spinal nerves to hindlimb innervation. In WT mice, segments L1-L3 contribute axons to the rostral lumbar plexus and segments L3-L5 contribute to the caudal sacral plexus (Fig. 3B). In the *Hoxc10*$^{-/-}$/*Hoxd10*$^{-/-}$ mutant mice, however, L1 and L2 did not project to the hindlimb, and instead appeared to innervate the body wall, and L3 and L4 contributed axons to the lumbar plexus and L4 and L5 contributed to the sacral plexus (n=6; 100% penetrance; Fig. 3B). At the level of the lumbar plexus, both the dorsal and ventral branches were present but significantly reduced in size, most likely because of the reduced number of segments projecting to this plexus. By contrast, the dorsal branch of the sacral plexus (the peroneal nerve), which normally supplies the anterior and lateral groups of muscles in the shank, was totally absent, whereas the ventral branch (the tibial nerve) was only slightly smaller than in WT embryos (Fig. 3B,C). The near total lack of innervation of anterior and lateral shank muscles, which normally extend and abduct the limb, could be a major reason for the crossed-limb phenotype observed in *Hoxc10*$^{-/-}$/*Hoxd10*$^{-/-}$ double-mutant mice.

In Hox10 triple-mutant embryos, the limb was innervated by even more caudal segments, with L4 and L5 contributing to the lumbar plexus and L5 and L6 contributing to the sacral plexus (data not shown). Surprisingly, the peroneal nerve was present in the triple mutants, although it was significantly smaller than normal (n=3). This may explain why the Hox10 triple-mutant animals seemed to have less severe locomotor defects than *Hoxc10*$^{-/-}$/*Hoxd10*$^{-/-}$ double mutants.

L1 and L2 motoneurons become thoracic motoneurons in *Hoxc10*$^{-/-}$/*Hoxd10*$^{-/-}$ double mutants

The above observations indicated that the L1 and L2 spinal nerves projected to the body wall rather than to the limb in *Hoxc10*$^{-/-}$/*Hoxd10*$^{-/-}$ mutants. This was confirmed by DiI injections into spinal cord segments L1 and L2 in double mutants (Fig. 4A). In addition, we observed many features consistent with the hypothesis that motoneurons in segments L1 and L2 actually differentiated into thoracic motoneurons in *Hoxc10*$^{-/-}$/*Hoxd10*$^{-/-}$ mutants, and never acquired characteristics of LMC neurons. For example, the columnar organization of motoneurons in the ventral horn of segments L1 and L2 resembled that of thoracic rather than lumbar spinal cord in double mutants (Fig. 4B). Normally at thoracic levels in WT animals, there are two motor columns in the spinal cord: the MMC in ventral cord, which has a medial and lateral division (mMMC and lMMC), and the visceral sympathetic preganglionic motor column (PGC) in the intermediolateral cord (Fig. 1A). By contrast, at lumbar levels motoneurons occupy the large LMC and smaller mMMC. In



B

Quantification of Isl1⁺ Motoneurons		
	Control	*c10*$^{-/-}$/*d10*$^{-/-}$
L3	180±45	178±46
L4	232±46	253±53
L5	289±55	287±32

Fig. 6. The lLMC is missing in *Hoxc10*$^{-/-}$/*Hoxd10*$^{-/-}$ double-mutant embryos, leading to defects in axon projection and inappropriate innervation. (**A**) Cross-sections of ventral spinal cord segments L1-L4 of control and double-mutant embryos stained for Isl1 to identify MMC and mLMC neurons and for Lim1 to identify lLMC neurons (dashed outlines). Note that Lim1$^+$ lLMC neurons are nearly eliminated in double-mutant embryos. (**B**) The number of Isl1$^+$ motoneurons counted unilaterally from L3 to L5 in control and *Hoxc10*$^{-/-}$/*Hoxd10*$^{-/-}$ mutant embryos (n=7 for each genotype). There was no significant difference between control and mutant embryos at E13.0-13.5; P>0.2 for each segment, t-test. (**C**) Cross-sections through lumbar segment L3 stained for Hb9 for motoneurons. Note that the number of Hb9$^+$ cells was markedly reduced in *Hoxc10*$^{-/-}$/*Hoxd10*$^{-/-}$ mutants. (**D**) DiI injected into spinal cord segments L3 and L4 labeled axons in the hindlimb. In control embryos, both the tibial (ventral view, arrow) and peroneal (dorsal, arrowhead) nerves were labeled. In double-mutant embryos, the ventral tibial nerve was present, but the peroneal nerve was missing. Some cutaneous axons were also labeled in dorsal views. (**E**) Cross-sections through ventral spinal cord segment L2 of control, and segment L3 of *Hoxc10*$^{-/-}$/*Hoxd10*$^{-/-}$ mutant embryos. In control embryos, tetramethylrhodamine dextran injected into the quadriceps muscle retrogradely labeled a subgroup of Lim1$^+$ lLMC neurons (arrow, yellow neurons) in L2, but did not label Isl1$^+$ mLMC neurons. By contrast, in double-mutant embryos, injection of dextran into the quadriceps retrogradely labeled Isl1$^+$ mLMC neurons in L3 (arrow). Scale bars: 100 μm in A,C; 50 μm in E.

Hoxc10$^{-/-}$/*Hoxd10*$^{-/-}$ mutants, however, staining of spinal cord sections for Isl1 and Lim3 showed that the arrangement of motor columns characteristic of thoracic cord extended caudally to the rostral part of L3 (Fig. 4B), with an apparent LMC first appearing only at L3. Furthermore, neuronal form of nitric oxide synthase (nNOS)$^{+}$/Isl1^{+} staining in intermediolateral cord, which is characteristic of visceral motoneurons (Thaler et al., 2004), extended caudally into rostral L3 in the double mutants (Fig. 4C). These ectopic nNOS^{+}/Isl1^{+} motoneurons behave like those in the thoracic region, projecting their axons to sympathetic ganglia (Fig. 4D). Thus, in *Hoxc10*$^{-/-}$/*Hoxd10*$^{-/-}$ mutants, motoneurons characteristic of thoracic cord were present in segments L1 and L2.

By contrast, markers characteristic of LMC neurons were absent from L1 and L2 in these mutant embryos. Retinaldehyde dehydrogenase 2 (Raldh2), a generic marker for LMC motoneurons, is expressed in LMC motoneurons throughout all lumbar segments in WT animals (Sockanathan and Jessell, 1998). In *Hoxc10*$^{-/-}$/*Hoxd10*$^{-/-}$ mutant animals, however, *Raldh2* expression was absent from segments L1 and L2 (Fig. 5A,A′), and was reduced in more caudal lumbar segments. Further evidence that LMC neurons were missing is that hindlimb muscles normally innervated by LMC neurons in these segments, such as the adductor (not shown) and quadriceps (Fig. 6E), were innervated by motoneurons in segment L3. Finally, several markers characteristic of specific LMC motor pools were not expressed in segments L1 and L2 in double mutants. For example, *Pea3* (Arber et al., 2000) and *Sema3E* (Livet et al., 2002; Messersmith et al., 1995) are normally expressed in several LMC motor pools at lumbar levels from L1 to L5, but not in thoracic regions. In double-mutant embryos, neither gene was expressed in segments L1 and L2, but both were still expressed in more caudal segments (Fig. 5B,B′ and data not shown). In mice, *ER81* is normally expressed in both thoracic (Fig. 5C,C′) and lumbar spinal cord (Arber et al., 2000), with a clear gap between the thoracic domain and the two lumbar pools (Fig. 5C,C′, Control). By contrast, in the double mutants, the thoracic domain of *ER81* expression extended into segments L1 and L2, and expression of *ER81* was lost in segments L3 and L4 (Fig. 5C,C′).

Because *Hox9* genes, especially *Hoxc9*, are suggested to be thoracic motoneuron determinants in chick (Dasen et al., 2003; Dasen et al., 2005), we asked whether altered expression of *Hoxc9* and *Hoxd9* in *Hoxc10*$^{-/-}$/*Hoxd10*$^{-/-}$ mutants could account for the switch of L1 and L2 motoneurons to a thoracic identity. In WT embryos, *Hoxc9* and *Hoxd9* were expressed in thoracic and lumbar spinal cord, with expression terminating around lumbar segment L5. There was no obvious change in expression of either *Hoxc9* or *Hoxd9* in the double knockouts (see Fig. S2 in the supplementary material; data not shown) (Carpenter et al., 1997). *Hoxc11* and *Hoxd11* transcripts were observed from L3 through the sacral segments in control embryos, and expression of these Hox genes was also not obviously altered in the double mutants (see Fig. S3 in the supplementary material; data not shown) (Tarchini et al., 2005).

Taken together these data show that motoneurons in segments L1 and L2 in *Hoxc10*$^{-/-}$/*Hoxd10*$^{-/-}$ double mutants differentiate as thoracic, rather than lumbar, motoneurons. Moreover, these findings identify *Hoxc10* and *Hoxd10* as having important roles both in establishing the border between thoracic and lumbar segments of spinal cord, and in defining the identity of motoneurons in these segments.

Lim1^{+} lateral LMC motoneurons are absent in segments L3-L5 of double mutants

Whereas motoneurons from L1 and L2 were converted to thoracic phenotypes, motoneurons from L3-L5 maintained their LMC identity, defined by *Raldh2* expression, and innervated the hindlimb in double mutants. However, neurons in segments L3-L5 are clearly impacted by deletion of *Hoxc10* and *Hoxd10* function. Since most hindlimb muscles received some innervation (Fig. 3A and see Fig. S1 in the supplementary material), motoneurons in segments L3-L5 must have taken over some functions normally mediated by neurons in L1 and L2. Despite this, there appeared to be fewer LMC neurons in L3-L5 in double mutants; *Raldh2* (Fig. 5A′) and Hb9 (Fig. 6C) expression were greatly reduced, and the peroneal nerve was missing entirely (Fig. 3B,C). To resolve these discrepancies, with an eye toward elucidating additional functions of Hox10 genes in motoneuron differentiation, we examined the subtype identity of LMC neurons in double mutants. Surprisingly, lLMC motoneurons, as defined by Lim1 staining, were severely reduced or absent in eight out of ten double-mutant embryos at E13.5, and noticeably reduced in the other two embryos (Fig. 6A). Lim1^{+} lLMC neurons were also not observed in lumbar segments at earlier stages (see Fig. S5 in the supplementary material), indicating that Lim1^{+} LMC neurons failed to differentiate in double mutants, rather than having differentiated and died. Instead, neurons in ventrolateral spinal cord, the usual location of the lLMC, expressed Isl1, characteristic of mLMC, and *Pea3* was restricted to Isl1^{+} neurons, instead of being expressed in both Lim1^{+} and Isl1^{+} motoneurons (Fig. 5B′ and data not shown), as in WT mice (Arber et al., 2000; Wang and Scott, 2007).

The number of Hb9^{+} neurons was reduced (Fig. 6C and see Fig. S6 in the supplementary material), demonstrating a paucity of LMC neurons. However, the number of mLMC neurons in L3-L5 was the same in control and *Hoxc10*$^{-/-}$/*Hoxd10*$^{-/-}$ mutants, based on counts of Isl1^{+} cells in serial sections of seven embryos of each genotype (Fig. 6B). Similarly, the number of Lim3^{+} neurons was not obviously affected in double mutants (data not shown). Thus, the missing Lim1^{+} motoneurons do not appear to have become Isl1^{+} or Lim3^{+} neurons. Instead, the lLMC appears to be missing entirely, with Isl1^{+} mLMC neurons being displaced to the most lateral part of the spinal cord in its absence. By contrast, the lLMC appeared to form normally in embryos homozygous for the individual new *Hoxc10* or *Hoxd10* mutant alleles (see Fig. S4 in the supplementary material).

Because Lim1^{+} lLMC motoneurons normally project their axons to dorsally derived hindlimb muscles in WT animals, the loss of the Lim1^{+} LMC neurons is most likely responsible for the absence of the peroneal nerve (Fig. 3B,C). This differs from the loss of the peroneal nerve in EphA4 mutants, which results from misrouting of lLMC neurons into ventral branches (Helmbacher et al., 2000). We verified that neurons in segments L3-L5 did not project in any dorsal nerve by injecting DiI into motoneurons in these segments (Fig. 6D). Thus, the loss of the Lim1^{+} lLMC neurons explains the lack of innervation in anterior or lateral shank muscles, which are normally innervated by axons in the peroneal nerve (Fig. 3A and see Fig. S1 in the supplementary material).

Intriguingly, however, dorsal thigh muscles, which are also normally innervated by Lim1^{+} lLMC neurons projecting in a dorsal nerve, clearly received some innervation (Fig. 3A) even though their usual motor pools appeared to be missing. To determine which neurons supplied dorsal thigh muscles, we retrogradely labeled quadriceps motoneurons with tetramethylrhodamine dextran. As expected (McHanwell and Biscoe, 1981), the quadriceps in WT

animals was innervated by Lim1$^+$/Isl1$^-$ lLMC motoneurons in segment L2, with a smaller contribution from L1. By contrast, quadriceps muscles in double-mutant embryos were innervated by Lim1$^-$/Isl1$^+$ mLMC motoneurons in segment L3 (Fig. 6E). Thus, in the absence of *Hoxc10* and *Hoxd10* function some mLMC motoneurons became misrouted and innervated dorsal-derived thigh muscles. The aberrant innervation of extensor muscles in the thigh, such as the quadriceps, by motoneurons that normally innervate flexor muscles in WT animals most likely contributed significantly to the locomotor defects in double mutants. If motoneurons receive their usual complement of central connections, as occurs when they innervate inappropriate muscles following surgical manipulations (Landmesser and O'Donovan, 1984; Vogel, 1987), then both extensors and flexors would be activated at the same time, leading to the rigid extended posture of limbs in mutant animals.

Motoneurons are generated on schedule and in normal numbers in the double mutants

There are a number of possible explanations for the absence of lLMC neurons in *Hoxc10*$^{-/-}$/*Hoxd10*$^{-/-}$ double mutants. To elucidate the functions of Hoxc10 and Hoxd10 during normal development, it was important to determine whether these gene products affect the initial generation of motoneurons, or are required for subsequent steps in their differentiation, migration and/or survival. To this end, we examined whether the earliest stages of motoneuron generation were compromised in *Hoxc10*$^{-/-}$/*Hoxd10*$^{-/-}$ mutants. Both the specification of motoneuron precursors and generation of postmitotic motoneurons appeared normal in double-mutant embryos. There was no obvious change in expression of Olig2, a marker for motoneuron progenitors, at E10.0-E10.5 at any segmental level in the double mutants (Fig. 7A). Moreover, there were no differences in Ngn2 and Nkx6.1 expression, two additional markers for ventral progenitors, between control and mutant embryos (data not shown). Isl1 is initially expressed by all newly generated postmitotic motoneurons in WT animals as they emerge from the ventricular zone, although its expression is subsequently lost in lLMC motoneurons (Arber et al., 1999; Ericson et al., 1992; Thaler et al., 2004). As with motoneuron progenitors, the numbers of newly generated Isl1$^+$ postmitotic motoneurons were similar in control and double knockout embryos at early stages (E10.0-E11.0; Fig. 7B). These observations suggest that *Hoxc10* and *Hoxd10* function are dispensable for the generation and initial specification of motoneurons during early embryogenesis.

Late-born motoneurons survive, but are misplaced in double mutants

The striking reduction in lLMC neurons in double mutants was not brought about by increased apoptosis. TUNEL staining of serial sections of lumbar spinal cord was nearly identical in controls and double mutants at all ages examined (E10.5-E14.0; see Fig. S6 in the supplementary material). This differs from a previous study in which increased apoptosis of neurons was suggested as a reason for forelimb locomotor defects in *Hoxc8* mutants (Tiret et al., 1998).

Motoneurons were initially generated in normal numbers and did not die in excess of normal, yet the entire Lim1$^+$ lLMC was absent in double mutants. Where are these missing neurons? To address this question, we compared the fate of *Hoxd10*-expressing cells, the cells we expected to be most directly affected by loss of the Hoxd10 gene product, in control and double-mutant animals. Because the *Hoxd10* mutant allele was generated by replacing the first exon of the *Hoxd10* gene with the *hrGFP* gene, we could follow the fate of cells in the double mutants by analyzing *hrGFP* expression in sections of heterozygous and double-mutant embryos. At E13.5 in *Hoxc10*$^{+/-}$/*Hoxd10*$^{+/-}$ heterozygous control embryos, *hrGFP*$^+$ motoneurons were located in the most lateral part of ventral horn (Fig. 8A), closely resembling the pattern of endogenous *Hoxd10* expression in the lLMC in WT embryos (Fig. 1E). By contrast, in the double-mutant mice, *hrGFP*$^+$ cells were no longer tightly clustered laterally, but instead were scattered throughout the entire ventral horn area (Fig. 8A). This finding suggests that the inactivation of *Hoxc10* and *Hoxd10* alters migration of *Hoxd10*-expressing cells in lumbar spinal cord.

The altered distribution of *Hoxd10*-expressing cells in the ventral horn raised the possibility that the missing lLMC motoneurons had changed their identity, but the numbers of motoneurons in the mLMC and mMMC had not increased in double mutants (Fig. 6B) and the numbers of Hb9$^+$ cells had decreased (Fig. 6C). Further, there was no obvious change in the expression of Chx10, a V2 interneuron marker (Arber et al., 1999; Ericson et al., 1997) in double mutants (data not shown). Instead, it appeared that cells fated to be lLMC neurons were born in normal numbers, but failed to acquire or retain markers characteristic of other populations of mature motoneurons or interneurons.

Motoneuron generation starts at E10.0 at the hindlimb level and is mostly completed by E11.0. Prospective lLMC motoneurons exit the cell cycle later than prospective mLMC motoneurons. These late-born motoneurons emerge from the ventricular zone, migrate laterally past the earlier-born mLMC motoneurons, acquiring their lLMC identity during the migration process, and eventually settle in the lateral part of the ventral horn (Sockanathan and Jessell, 1998). The difference in timing of generation of mLMC and lLMC neurons allowed us to investigate the fate of the late-born motoneurons in more detail. We labeled late-born cells by injecting BrdU into pregnant females at E10.5, a time by which the early-born motoneurons in WT embryos have already exited cell cycle and no longer incorporate BrdU, and analyzed motoneuron identity and distribution at E12.0. In the control embryos, most BrdU$^+$ cells in the ventral horn settled laterally and were Lim1$^+$/Isl1$^-$, suggesting that late-born cells were indeed lLMC motoneurons (Fig. 8B). By contrast, in double-mutant embryos, BrdU$^+$ cells were scattered throughout the ventral horn and intermingled with Isl1$^+$ cells.

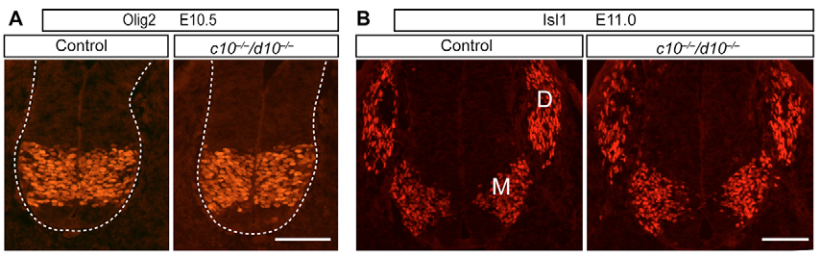


Fig. 7. Motoneurons are generated normally in *Hoxc10*$^{-/-}$/*Hoxd10*$^{-/-}$ double-mutant embryos. Cross-sections of lumbar spinal cord of control and double-mutant embryos stained for (**A**) Olig2, a marker for motoneuron progenitors, and (**B**) Isl1 to label newly generated postmitotic motoneurons. There were no apparent differences in the numbers of Olig2$^+$ motoneuron progenitors or Isl1$^+$ motoneurons between control and double-mutant embryos. D, dorsal root ganglion; M, motoneurons. Scale bars: 100 μm.

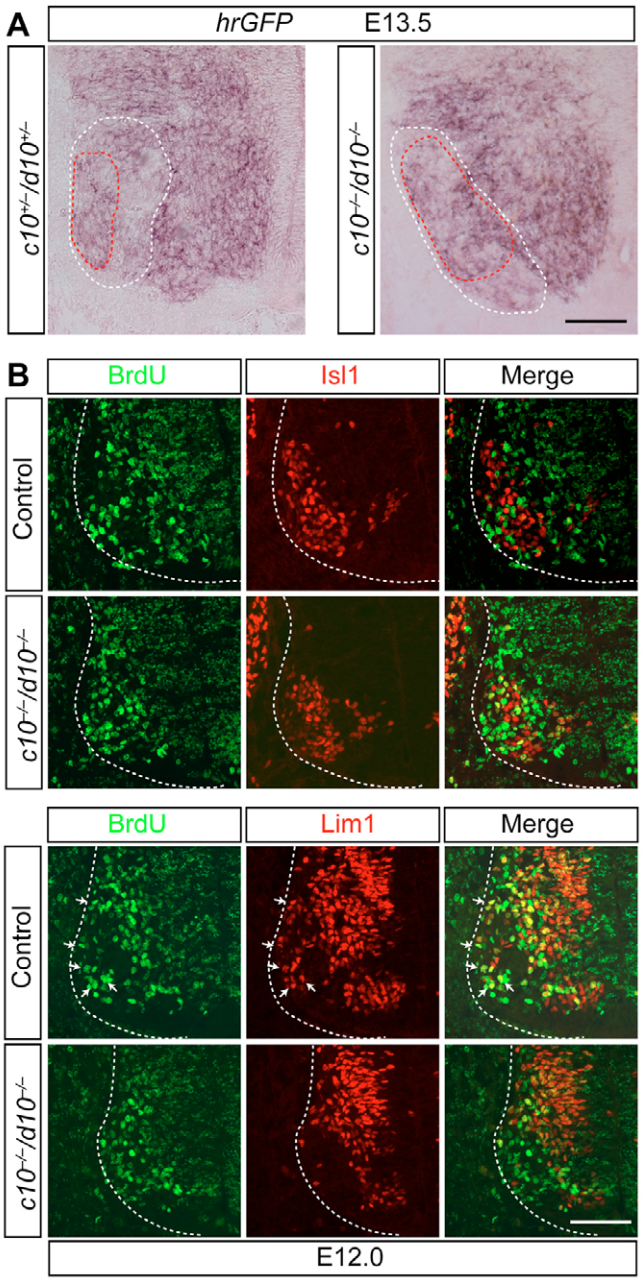


Fig. 8. lLMC neurons are generated normally, but fail to migrate laterally, and are scattered throughout ventral horn in *Hoxc10*$^{-/-}$/*Hoxd10*$^{-/-}$ double-mutant embryos. (**A**) Cross-sections of the ventral spinal cord of *Hoxc10*$^{+/-}$/*Hoxd10*$^{+/-}$ and *Hoxc10*$^{-/-}$/*Hoxd10*$^{-/-}$ embryos processed with an *hrGFP* in situ probe. In control embryos, *hrGFP* expression closely resembles *Hoxd10* expression (see Fig. 1E). In *Hoxc10*$^{-/-}$/*Hoxd10*$^{-/-}$ mutant embryos, however, *hrGFP*$^{+}$ cells are scattered throughout the ventral horn. Dashed white lines outline the LMC; dashed red lines outline the region of the LMC that contains *hrGFP*$^{+}$ cells. (**B**) To examine the fate of late-born motoneurons, timed pregnant animals were injected with BrdU at E10.5 and analyzed at E12.0. Cross-sections of the ventral spinal cord were labeled with anti-BrdU and either anti-Isl1 (top panels) or anti-Lim1 (bottom panels). In control embryos, most late-born BrdU^{+} motoneurons are laterally migrating Lim1^{+} lLMC neurons (arrows indicate double-labeled cells), whereas in *Hoxc10*$^{-/-}$/*Hoxd10*$^{-/-}$ double-mutant embryos, most late-born BrdU^{+} motoneurons expressed neither Isl1 nor Lim1 and were scattered in ventral horn, intermixed with earlier-born Isl1^{+} motoneurons. Scale bars: 100 μm.

Importantly, most BrdU^{+} cells expressed neither Isl1 nor Lim1. Thus, the loss of Hoxc10 and Hoxd10 appears to cause late-born motoneurons to differentiate incompletely. These neurons downregulated expression of Isl1 on schedule, but failed to migrate to their appropriate location or acquire other markers characteristic of mature motoneurons, consistent with the observed decrease in Hb9^{+} cells. It is possible that some of the late-born motoneurons in double mutants differentiated into interneurons, but investigation of this possibility must await discovery of additional markers of mature interneurons.

DISCUSSION

During embryonic development, motoneurons become patterned with respect to their columnar, divisional and pool identities, which enables them to establish connections with the appropriate peripheral target muscles with remarkable precision (Jessell, 2000; Landmesser, 2001). Here we show that *Hoxc10*, which had not been previously studied, and *Hoxd10* together play essential roles in patterning lumbar motoneurons at all three levels of organization, and elucidate some of the cellular and molecular processes governed by these genes. We also show that these genes have minor, but consistent, effects on patterning hindlimb muscles.

Hoxc10 and Hoxd10 determine the rostral boundary of lumbar motor columns

Our analysis of *Hoxc10*$^{-/-}$/*Hoxd10*$^{-/-}$ double-mutant embryos showed conclusively that Hox10 gene products govern the patterning of lumbar versus thoracic motor columns. Motoneurons in segments L1 and L2 differentiated as thoracic motoneurons in double mutants, expressing nNOS, but failing to express markers of LMC neurons, such as *Raldh2*. The remaining LMC in segments L3-L5 innervated the entire hindlimb, indicating that the LMC in *Hoxc10*$^{-/-}$/*Hoxd10*$^{-/-}$ mutants was compressed from five to three segments, rather than simply being shifted posteriorly, as suggested previously (Lin and Carpenter, 2003).

Several lines of evidence suggest that *Hoxc10* and *Hoxd10* play primary roles in the patterning of thoracic versus lumbar motor columns within the spinal cord, with *Hoxa10* playing a lesser role. For example, *Hoxa10*, unlike *Hoxc10* and *Hoxd10*, is not expressed in prospective motoneurons during their early genesis, and therefore is unlikely to be involved in the early steps of their specification. Furthermore, locomotor defects were more severe in H*oxc10*$^{-/-}$/*Hoxd10*$^{-/-}$ mutants than in *Hoxa10*$^{-/-}$/*Hoxc10*$^{-/-}$ or *Hoxa10*$^{-/-}$/*Hoxd10*$^{-/-}$ mutants. Importantly, ectopic expression of *Hoxd10* in thoracic motoneurons in chick is sufficient to convert them into lumbar-like motoneurons (Shah et al., 2004). The conversion of prospective LMC neurons in L1 and L2 to thoracic motoneurons in the *Hoxc10*$^{-/-}$/*Hoxd10*$^{-/-}$ mutants may result from the persistence of *Hox9* gene function in these neurons in the absence of normal *Hoxc10* and *Hoxd10* expression [i.e. a case of elimination of posterior prevalence (Duboule and Morata, 1994)].

Thus, it appears that *Hoxc10* and *Hoxd10*, together with more rostrally expressed Hox genes, determine the rostral border between thoracic and lumbar motor columns in the spinal cord. The failure to convert more caudal segments to thoracic cord as well as the persistence of an LMC in more caudal segments in *Hoxc10*$^{-/-}$/*Hoxd10*$^{-/-}$ mutants is most likely due to the presence of *Hox11* genes, which are expressed in the caudal spinal cord from segment L3 in both WT (Carpenter, 2002) and *Hoxc10*$^{-/-}$/*Hoxd10*$^{-/-}$ mutant embryos (see Fig. S3 in the supplementary material). Interestingly, the LMC in caudal lumbar segments normally consists

predominantly of Isl1$^+$ mLMC neurons in both chick and mouse (data not shown), similar to the LMC in *Hoxc10*$^{-/-}$/*Hoxd10*$^{-/-}$ mutants. *Hox11* genes appear to be important in generating these motoneurons, since misexpression of *Hoxd11* in rostral lumbar motoneurons induces an overabundance of Isl1$^+$ mLMC neurons relative to Lim1$^+$ lLMC neurons (Misra et al., 2005). In summary, *Hoxc10* and *Hoxd10* are required for proper columnar specification of the lumbar motoneurons.

Hoxc10 and Hoxd10 determine divisional specification in the LMC

In addition to establishing the boundary between thoracic and lumbar motor columns, our results reveal a novel role for Hox10 genes in the divisional specification of LMC. *Hoxc10* and *Hoxd10* are essential for development of the lateral division of the LMC in lumbar cord. The lLMC was nearly eliminated in *Hoxc10*$^{-/-}$/*Hoxd10*$^{-/-}$ mutants as evidenced by the reduction or loss of lateral Lim1$^+$ neurons and the dorsal nerve branches of the lumbar and sacral plexii. Our results differ from the reported milder phenotype for *Hoxa10*$^{-/-}$/*Hoxd10*$^{-/-}$ mice, in which both divisions of LMC neurons were present in neonates but reduced in size; although the spatial relationships between the two groups of cells were retained, the groups were clustered together (Lin and Carpenter, 2003).

Perturbation of any number of developmental processes could produce a lack of lLMC neurons in *Hoxc10*$^{-/-}$/*Hoxd10*$^{-/-}$ double mutants. We show here that the loss of the lLMC did not result from a decreased production of motoneuron precursors or from the increased apoptosis of Lim1$^+$ motoneurons. Instead, presumptive lLMC neurons failed to migrate to their normal position and never acquired markers characteristic of known populations of motoneurons or interneurons. The observed migratory defect resembles the effects of perturbing cadherin expression on motoneuron sorting (Price et al., 2002), suggesting a mechanism by which Hox genes could govern migration of prospective lLMC neurons. Thus, in the absence of Hoxc10 and Hoxd10, motoneuron precursors appear to be generated normally, but late-born neurons fail to differentiate into lLMC neurons or into any other clearly recognizable neuron population.

Hoxc10 and Hoxd10 affect motor pool specification and limb muscle development

Motor pools represent specific groups of motoneurons in the LMC that establish functional connections with individual muscles in the limb. Our findings show that *Hoxc10* and *Hoxd10* influence lumbar motor pool formation, although these effects may be indirect as a consequence of Hox10 function in columnar and divisional specification. Motor pools were clearly aberrant in *Hoxc10*$^{-/-}$/*Hoxd10*$^{-/-}$ mutants. There was no LMC in segments L1 and L2 and no lLMC in more caudal segments in double-mutant embryos, yet most hindlimb muscles were innervated. The remaining Isl1$^+$ mLMC neurons in segments L3-L5 must, therefore, have distributed themselves among many more muscles than normal, clearly altering motor pools. Retrograde labeling showed that at least one muscle, the quadriceps, was innervated inappropriately by Isl1$^+$ neurons in the absence of Lim1$^+$ neurons. We expect that other dorsal muscles in the thigh were similarly innervated by Isl1$^+$ neurons, which normally innervate ventral muscles.

Further evidence that Hox10 genes influence motor pool formation is that the normal expression patterns of the ETS transcription factor genes, *ER81* and *Pea3*, which are restricted to specific motor pools in WT animals (Arber et al., 2000), were altered in *Hoxc10*$^{-/-}$/*Hoxd10*$^{-/-}$ mutant embryos (Fig. 5B,B′ and 5C,C′). Some change in ETS expression was expected, since some motoneurons that usually express ETS factors were missing in double mutants. In addition, innervation of inappropriate muscles by the remaining motoneurons may also contribute to altered expression of *ER81* and *Pea3*, since ETS expression is normally initiated and shaped by signals from the periphery (Lin et al., 1998; Wang and Scott, 2004). In addition, the peripheral signals themselves may be perturbed in double mutants.

Hox10 genes are expressed in the developing hindlimb (Morgan and Tabin, 1994; Nelson et al., 1996; Wellik and Capecchi, 2003) as well as in the lumbar spinal cord. Thus, the disruption of Hox10 genes in the periphery may have contributed to the observed perturbations in muscle innervation. For example, we have previously shown that restricted inactivation of *Hoxb1* in the periphery resulted in the failure of these motoneuron axons to innervate the facial muscles (Arenkiel et al., 2004). Therefore, the locomotor and innervation mutant phenotypes in the *Hoxc10*$^{-/-}$/*Hoxd10*$^{-/-}$ double mutants reported here are likely to have resulted from contributions of Hox function in both motoneuron specification and in motoneuron targeting in the periphery. These potential contributions should be separable through the use of conditional mutagenesis. Importantly however, the motoneuron specification defects discussed in this paper are not likely to have been affected by the functions of *Hoxc10* and *Hoxd10* in the periphery, since this specification occurs before the axons grow into the limb and indeed before the motoneurons are born (Matise and Lance-Jones, 1996).

In conclusion, we have elucidated novel functions of *Hoxc10* and *Hoxd10* in the patterning of lumbar motoneurons. We showed that disruption of *Hoxc10* and *Hoxd10* causes rostral lumbar motoneurons to adopt a thoracic phenotype, and prevents the differentiation of Lim1$^+$ lateral LMC neurons. Most hindlimb muscles in double mutants become innervated by the remaining medial LMC neurons. Together, these results show that *Hoxc10* and *Hoxd10* are important in establishing the columnar, divisional and motor pool identity of lumbar motoneurons. The downstream cascades of genes activated and repressed by Hoxc10 and Hoxd10, which ultimately govern the differentiation of lumbar motoneurons, remain to be determined.

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Supplementary material

Supplementary material for this article is available at
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