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LIM Homeodomain Factors Lhx3 and Lhx4 Assign Subtype Identities for Motor Neurons

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Summary

The circuits that control movement are comprised of discrete subtypes of motor neurons. How motor neuron subclasses develop and extend axons to their correct targets is still poorly understood. We show that LIM homeodomain factors Lhx3 and Lhx4 are expressed transiently in motor neurons whose axons emerge ventrally from the neural tube (v-MN). Motor neurons develop in embryos deficient in both Lhx3 and Lhx4, but v-MN cells switch their subclass identity to become motor neurons that extend axons dorsally from the neural tube (d-MN). Conversely, the misexpression of Lhx3 in dorsal-exiting motor neurons is sufficient to reorient their axonal projections ventrally. Thus, Lhx3 and Lhx4 act in a binary fashion during a brief period in development to specify the trajectory of motor axons from the neural tube.

Introduction

Appropriate motor neuron connectivity is established by generating subtypes of motor neurons during development. The distinct subclasses of motor neurons become apparent as their cell bodies settle in defined positions and their axons project along stereotyped pathways (Landmesser, 1978, 1992). Despite substantial information regarding the characteristics of motor neuron subtypes, the factors dictating the specification of this diverse ensemble of neurons remain largely unknown.

Sonic hedgehog (Shh), secreted from the notochord and floor plate, triggers MNR2 expression and motor neuron differentiation (Roelink et al., 1994; Marti et al., 1995; Tanabe et al., 1998). As motor neurons become postmitotic, they begin to express the LIM homeodomain (LIM-HD) transcription factor Isl1 (Ericson et al., 1992). This factor belongs to a family of evolutionarily conserved homeodomain proteins with LIM domains (Karlsson et al., 1990), which are zinc-binding motifs for interfacing with other proteins (Agulnick et al., 1996; Jurata et al., 1996; Bach et al., 1997). Genetic studies of LIM-HD genes such as *islet* (*Isl1/2* homolog) and *apterous* (*Lhx2* homolog) in *D. melanogaster* have shown that these factors are required for axonal fasciculation and pathfinding (Lundgren et al., 1995; Thor and Thomas, 1997).

Whereas induction by Shh and expression of Isl1 are generically required for motor neuron differentiation (Chiang et al., 1996; Pfaff et al., 1996), studies in the chick and zebrafish have identified four LIM-HD factors that combinatorially mark different types of motor neurons (Isl1, Isl2, Lim1, and Lim3) (Tsuchida et al., 1994; Appel et al., 1995; Varela-Echavarria et al., 1996). In the chick, these motor neuron subtypes correspond to classes of cells that sort into columns within the spinal cord and extend axons along different major pathways. By grafting young neural tube cells into new anterior–posterior locations, the subtype identity of motor neurons can be modified (Eisen, 1991; Matise and Lance-Jones, 1996). This change in motor neuron identity is accompanied by a coordinate change in LIM-HD expression, indicating that these factors may control the columnar identity of motor neuron subtypes (Appel et al., 1995; Ensini et al., 1998).

In addition to the columnar organization of motor neuron subtypes, these cells can be subdivided based on such differences as their pool organization in the spinal cord, the dorsal–ventral location from which their axons emerge from the neural tube (v-MN or d-MN, see below), and the cell type they innervate (somatic or visceral) (Tanabe and Jessell, 1996; Pfaff and Kintner, 1998). Whether LIM-HD factors contribute to the specification of these discrete properties remains unclear for two reasons. First, during the critical period in which motor neuron identities are established, individual subtypes of cells are generated together and are intermingled (Nornes and Carry, 1978; Leber et al., 1990; Leber and Sanes, 1995). And second, LIM-HD gene expression patterns appear to change rapidly in motor neurons during the initial period in which they are generated (Tsuchida et al., 1994; Appel et al., 1995). Thus, it is unclear what the LIM-HD gene codes actually are in specific motor neuron subtypes prior to axon extension and what functional role these codes may play.

In this study we have defined the expression of two related LIM-HD genes, *Lhx3* (*Lim3, pLim*) and *Lhx4* (*gsh4*) (Singh et al., 1991; Bach et al., 1995; Zhadanov et al., 1995), during motor neuron development, using CRE-mediated lineage tracing in the mouse. We found that these factors have an extremely dynamic expression pattern. For a brief period, as motor neurons are born, Lhx3 and Lhx4 are expressed in all motor neuron classes that extend axons ventrally from the neural tube. In contrast, motor neurons that send axons dorsally from

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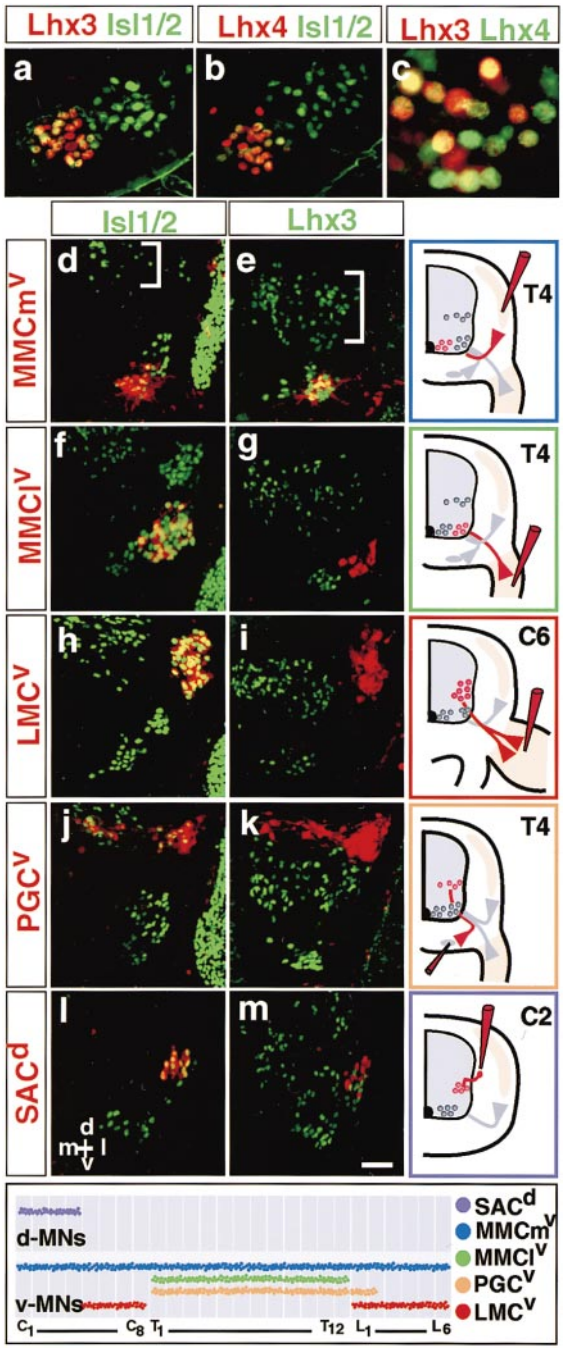

Figure 1. Coexpression of Lhx3 and Lhx4 in the MMCm^{v} Motor Column

(A–E) Immunocytochemical detection of Lhx3, Lhx4, and Isl1/2 in the right ventral quadrant of an e13.5 mouse thoracic spinal cord. (A and B) Coexpression (red-yellow) of (A) Lhx3 (red) and (B) Lhx4 (red) with Isl1/2 (green) in a medial subset of motor neurons. (C) Medial motor neurons coexpress Lhx3 (red) and Lhx4 (green) at varying levels. (D–M) Rhodamine-dextran motor column backlabeling (red) combined with immunocytochemical detection of LIM-HD factors (green) in e13.5 mice. Schematics summarize the axonal projections and spinal levels of motor columns. Dorsal exiting motor neurons (d-MN) are represented by SAC^{d} (purple), whereas ventral-exiting motor neurons are represented by MMCm^{v} (blue), MMCl^{v} (green), PGC^{v} (yellow), and LMC^{v} (red). Lhx3 and Lhx4 are coexpressed (A–C); therefore, only Lhx3 is shown with retrograde labeling. (D and E) Medial motor neurons of the medial motor column (MMCm^{v}) innervating dorsal axial muscles express Isl1/2, Lhx3, and Lhx4. Brackets in (D) and (E) indicate Isl1^{+} and Lhx3^{+} interneurons, respectively. (F and G) Lateral motor neurons of the medial motor column (MMCl^{v}) innervating ventral body wall muscles express Isl1/2 but do not express Lhx3 or Lhx4. (H and I) Lateral motor column cells (LMC^{v}) projecting into the dorsal and ventral limb express Isl1/2 but do not express Lhx3 or Lhx4. (J and K) Preganglionic motor column cells (PGC^{v}) projecting to the sympathetic ganglia express Isl1/2 but do not express Lhx3 or Lhx4. (L and M) Spinal accessory motor column cells (SAC^{d}) projecting dorsally out the neural tube to innervate neck and shoulder muscles express Isl1/2 but do not express Lhx3 or Lhx4. Note that Lhx3^{+} (green) interneurons in (M) are not backlabeled, but they intermingle with SAC^{d} motor neurons (red). Dorsal–ventral, medial–lateral orientations for all panels indicated in (L).

Scale bar in (M) corresponds to 40 μm in (A–B), 8 μm in (C), and 60 μm in (D–M).

the neural tube (d-MNs) arise from cells that do not express Lhx3 and Lhx4 as they are born. Following v-MN birth, Lhx3 and Lhx4 become restricted to a single motor column.

To test whether these LIM-HD factors specify v-MN or motor column identity, we examined *Lhx3*$^{-/-}$, *Lhx4*$^{-/-}$, and *Lhx3*$^{-/-}$ and *Lhx4*$^{-/-}$ (double mutant, DKO) knockout mice. In DKO mice, motor neuron differentiation proceeds; however, v-MN cells acquire properties of d-MNs. Moreover, elimination of *Lhx3* or *Lhx4* alone does not produce this phenotype, indicating that these factors have similar activities in motor neurons. Finally, we show that Lhx3 is sufficient to specify v-MN identity when misexpressed in progenitors for d-MN cells. Our studies demonstrate that Lhx3 and Lhx4 act independently from the factors that trigger motor neuron differentiation to control the choice of motor neuron axon exit point from the neural tube.

Results

MMCm^{v} Motor Neurons Express Lhx3 and Lhx4

To examine the role of LIM-HD genes in motor neuron development, we elected to use molecular-genetic approaches in mice. Since the combinatorial expression of LIM-HD genes has not been well defined in mouse motor neurons, we first generated antibodies to detect murine LIM-HD factors Isl1, Isl2, Lhx3, and Lhx4. Together, Isl1 and/or Isl2 (Isl1/2) appear to mark all motor neurons (Figure 1) and a group of dorsally located interneurons (see Figure 1D). Motor neuron subtypes become evident by e13.5 in mouse development as axons arrive at their appropriate targets and cell bodies settle in their columnar locations in the neural tube. At this stage, Lhx3 and Lhx4 are coexpressed, to varying degrees, in a medial subset of Isl1/2^{+} motor neurons (Figures 1A–1C) and a group of ventrally located interneurons (see Figure 1E). In the chick, *Lhx3*(*Lim3*) is also detected in medial motor neurons and interneurons (Tsuchida et al., 1994; Ericson et al., 1997).

The positions of embryonic mouse motor columns have not been characterized, making it difficult to accurately assign LIM-HD gene expression to specific subclasses of motor neurons. To precisely define the identity of the medial Lhx3^{+} and Lhx4^{+} cells, retrograde

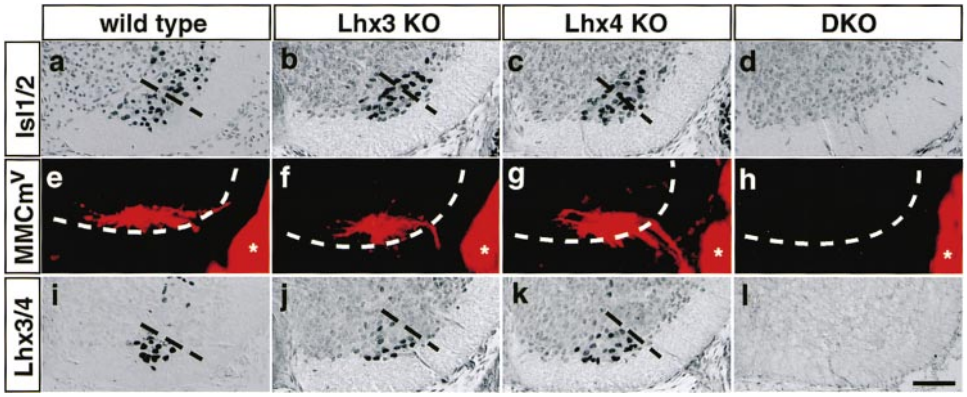

Figure 2. Knockouts of Lhx3 and Lhx4 Reveal a Critical Function in Motor Neuron Differentiation

Thoracic motor neurons in the right-ventral quadrant of e13.5 mouse embryos.

(A–D) Isl1/2 immunocytochemical staining of MMCm^v and MMCl^v motor neurons in the ventral horn of (A) wild-type, (B) *Lhx3* knockout (KO), and (C) *Lhx4* KO embryos. The dotted line in (A–C) and (I–K) divides MMCm^v (left side) from MMCl^v (right side). Isl1/2$^+$ motor neurons are present in normal numbers and locations in knockouts of either *Lhx3* or *Lhx4*. (D) Double knockouts (DKO) deficient in both Lhx3 and Lhx4 lack Isl1/2$^+$ MMCm^v and MMCl^v cells.

(E–H) Retrogradely transported DiI from injections into dorsal axial muscles labels MMCm^v motor neurons (see Figure 1) and dorsal root ganglia neurons (*) in (E) wild-type, (F) *Lhx3* KO, and (G) *Lhx4* KO embryos. (H) In DKO embryos, sensory neurons (*) are backlabeled, but no MMCm^v motor neurons are detected.

(I and J) Immunocytochemical staining to detect Lhx4 expression in (I) wild-type and (J) *Lhx3* KO embryos.

(K and L) Immunocytochemical staining to detect Lhx3 expression in (K) *Lhx4* KO and (L) DKO embryos. No Lhx3 (data not shown) or Lhx4 is detected in DKO embryos.

Scale bar in (L) corresponds to 70 μm in all panels.

labeling with rhodamine-conjugated dextran was used to mark individual motor columns. We first defined the location of motor neurons that project axons ventrally from the neural tube (v superscript) and examined their LIM-HD gene expression. Motor neurons projecting to axial muscles are located in the medial portion of the medial motor column (MMCm^v) and express Isl1, Isl2, Lhx3, and Lhx4 (Figures 1A–1E). In contrast, neither Lhx3 nor Lhx4 was detected in Isl1/2$^+$ motor neurons projecting to body wall muscles (MMCl^v, Figures 1F and 1G), limb muscles (LMC^v, Figures 1H and 1I), or the sympathetic ganglia (PGC^v, Figures 1J and 1K). Next we examined motor neurons whose axons exit dorsally from the neural tube (d superscript). We found that Isl1/2$^+$ spinal accessory motor neurons (SAC^d), which innervate neck and shoulder muscles, do not express Lhx3 and Lhx4 (Figures 1L and 1M). Although the locations of particular motor columns differ between mouse and chick, the demarcation of motor columns by coexpressed LIM-HD factors is conserved (Tsuchida et al., 1994). We find that, in motor neurons, Lhx3 and Lhx4 are restricted to the MMCm^v at e13.5.

Lhx3 and Lhx4 Coordinately Control the Differentiation of MMCm^v Motor Neurons

The restricted expression of Lhx3 and Lhx4 in MMCm^v motor neurons raised the possibility that these factors may control the differentiation, axonal projections, and/or columnar organization of this subclass of motor neurons. Previous studies to examine the function of *Lhx3* and *Lhx4* in the pituitary have used homologous recombination in mouse embryonic stem cells to create mice with null mutations in these genes (Li et al., 1994; Sheng et al., 1996, 1997). In embryos lacking either *Lhx3* alone or *Lhx4* alone, we find that MMCm^v motor neurons differentiate normally (Figures 2A–2C), send axons to their appropriate targets (Figures 2E–2G), and settle into their normal ventral–medial positions (Figures 2E–2G and 2I–2K).

Three observations suggested that we should examine mice lacking both Lhx3 and Lhx4. First, Lhx3 and Lhx4 have 95% amino acid identity in their homeodomains and ~70% overall identity (Li et al., 1994; Zhadanov et al., 1995; Yamashita et al., 1997). Second, these LIM-HD factors are coexpressed (Figures 1A–1C); and third, mice deficient in either Lhx3 or Lhx4 continue to express Lhx4 or Lhx3, respectively (Figures 2J and 2K). Intercrosses of compound heterozygous mice (*Lhx3*$^{+/-}$, *Lhx4*$^{+/-}$) produced ~1 in 16 progeny that lacked both functional alleles for *Lhx3* and *Lhx4*. Accordingly, these double knockout embryos (DKO) failed to express immunocytochemically detectable Lhx3 and Lhx4 (Figure 2L; data not shown). The overall appearance of DKO embryos was indistinguishable from their littermates, though they died at birth (data not shown) (Sheng et al., 1997). In contrast to single knockouts of *Lhx3* or *Lhx4*, DKO embryos lack MMCm^v motor neurons (Figures 2D and 2H). Unexpectedly, other classes of Isl1/2$^+$ motor neurons, such as the MMCl^v, were also found to be absent in DKO embryos (Figure 2D, see below).

Expression of Lhx3 in the v-MN Lineage

Because the loss of motor neurons in DKO embryos was not restricted to the MMCm^v column as predicted from the expression patterns of these LIM-HD genes observed at e13.5, we reasoned that Lhx3 and Lhx4 could be transiently expressed in additional motor column subtypes prior to their sorting and axon extension. Lhx3$^+$ and Lhx4$^+$ cells are first detected near the ventricular zone at ~e9.5 in a subset of dividing cells labeled by the M phase marker mpm2, just prior to the initiation

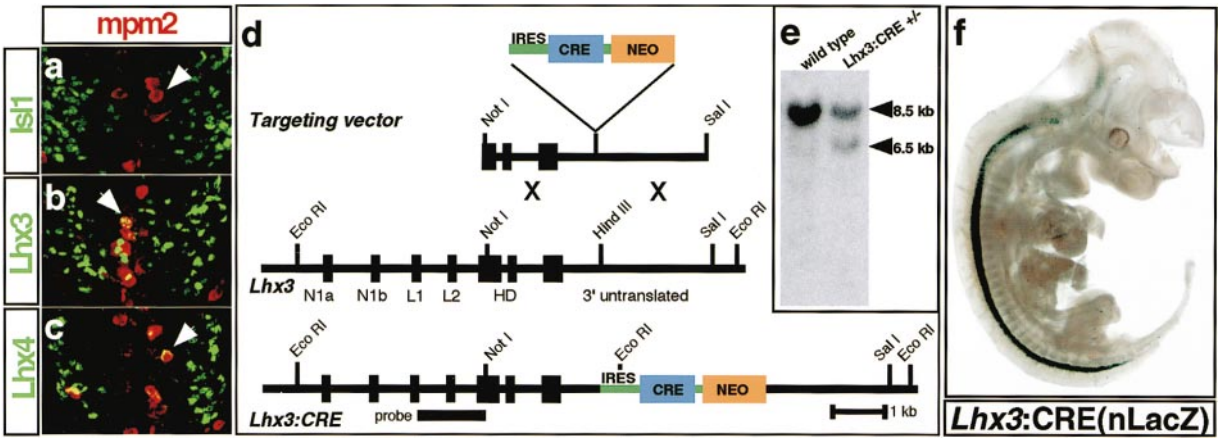

Figure 3. CRE Lineage Tracing of Lhx3$^+$ and Lhx4$^+$ Progenitor Cells

(A–C) (A) Isl1/2 (green) is not detected in mouse e9.5 M phase cells labeled by mpm2 (red, arrowhead) in the ventricular zone. (B) Lhx3 and (C) Lhx4 are coexpressed in mpm2-labeled cells (punctate yellow, arrowheads).

(D and E) (D) Schematic representation of the targeting strategy used to insert *CRE* into the 3′ untranslated region of *Lhx3*. An internal ribosome entry site (*IRES*) and a neomycin resistance gene (*NEO*) were included with *CRE*. (E) Southern blot analysis of EcoRI-digested DNA reveals a wild-type 8.5 kb and recombinant 6.5 kb band with the probe indicated in (D).

(F) X-Gal staining of an e11.5 F1 embryo carrying the *Lhx3:CRE* allele and an *nLacZ reporter* for CRE. nLacZ staining is detected in the ventral region of the spinal cord and hindbrain but not in other peripheral tissues, consistent with the normal expression pattern of Lhx3.

of Isl1 expression in postmitotic motor neurons (Figures 3A–3C). However, the fate of these Lhx3/4$^+$ cells is unclear, as motor neuron subtype identity cannot be assigned to particular cells prior to axon extension. To determine the identity of the lineal descendants of Lhx3$^+$ and Lhx4$^+$ precursors, we used CRE DNA recombinase to indelibly mark cells for characterization of their eventual identity (Sauer, 1993; Zinyk et al., 1998). Since *Lhx3* and *Lhx4* have overlapping expression patterns (Figures 1A–1C) and redundant functions (Figure 2), we focused our analysis on a single gene, *Lhx3*. Homologous recombination in mouse embryonic stem (ES) cells was used to insert *CRE* into the 3′ untranslated region of the *Lhx3* gene (*Lhx3:CRE*) (Figures 3D and 3E). This strategy is intended to encode CRE and Lhx3 from a bicistronic mRNA. To follow the progeny of Lhx3$^+$ precursor cells, *Lhx3:CRE* mice were mated to *CRE-reporter* mice that activate expression of nuclear-β-galactosidase (nLacZ) following CRE-mediated DNA recombination (Tsien et al., 1996; Zinyk et al., 1998). Embryos with *Lhx3:CRE* and *CRE-reporter* alleles from this mating (termed F1 embryos) showed normal Lhx3 expression (data not shown), and the overall pattern of CRE activity reflected the embryonic pattern of *Lhx3* expression (Figure 3F) (Taira et al., 1993; Seidah et al., 1994; Zhadanov et al., 1995). These observations suggest that CRE accurately reports the expression of *Lhx3* in *Lhx3:CRE* mice.

F1 embryos with *Lhx3:CRE* and *CRE-reporter* alleles were examined for nLacZ expression at e13.5 to identify motor neuron subtypes that descend from Lhx3$^+$ precursors. As expected, Lhx3$^+$ MMCm^v motor neurons were found to be nLacZ$^+$ in F1 embryos (Figure 4A). As hypothesized, we found that several Isl1/2$^+$ motor columns were nLacZ$^+$ through Lhx3$^-$ at e13.5 (compare Figures 4B and 1I). This was an indication that Lhx3 is expressed in many subtypes of motor neurons as they are generated from progenitor cells (Figures 3A–3C). Backlabeling in F1 embryos showed that somatic MMCl^v, somatic LMC^v, and visceral PGC^v cells were marked by nLacZ (Figure 4B; data not shown). Since we only detected nLacZ in postmitotic cells (data not shown), it is unlikely that the nLacZ$^+$ motor columns in F1 embryos inherited the activated reporter from an early embryonic Lhx3$^+$ cell lineage. The 20%–50% activation of the nLacZ reporter in these labeled motor columns did, however, suggest that the level of CRE expressed by internal translation initiation is limiting for recombination in motor neurons.

As we found that motor columns comprised of cells that extend axons ventrally (v-MN) from the neural tube were nLacZ$^+$, we next tested whether dorsal-exiting motor neurons (d-MN) were labeled by nLacZ in F1 embryos. In contrast to v-MN motor columns, no nLacZ was detected in the d-MN class of SAC^d motor neurons (Figure 4C). The nLacZ reporter can, however, be activated and expressed in SAC^d cells, as shown by mating nLacZ reporter mice to a *Pro:CRE* mouse line (Figure 4C, inset) (O'Gorman et al., 1997). The expression of CRE in the male germ line of *Pro:CRE* mice leads to inheritance of an activated reporter in all tissues.

To test more generally if v-MN motor classes are nLacZ$^+$ and d-MN motor classes are nLacZ$^-$ in F1 embryos, we examined the hindbrain, where a variety of d-MN and v-MN motor classes form in stereotyped locations. We found that v-MN motor neurons, such as the hypoglossal (XII^v, somatic) and abducens (VI^v, somatic), were nLacZ$^+$ (Figure 4D; data not shown), whereas d-MNs, such as the trigeminal (V^d, visceral) and trochlear (IV^d, somatic), were not marked by nLacZ (Figures 4E and 4F). In contrast to Lhx3 expression at e13.5, CRE reveals that early Lhx3 expression is not restricted to cells that give rise to a single motor column, nor is it restricted to only somatic or visceral cells. Our results demonstrate that early Lhx3 expression occurs in v-MN cells, but evidence of Lhx3 expression in d-MN cells was never detected.

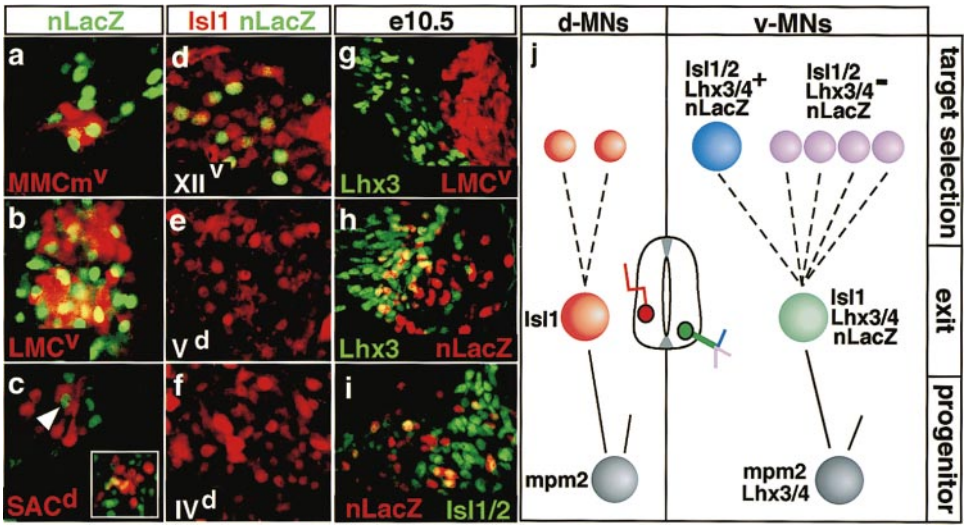

Figure 4. v-MN Cells Transiently Express Lhx3

(A–C) Immunocytochemical localization of nLacZ (green) in motor neurons (red) in F1 e13.5 embryos with *Lhx3:CRE* and *CRE-reporter* genes. Rhodamine-dextran backlabeling (red; see Figure 1) of (A) MMCm^{v} and (B) LMC^{v} shows colabeling (yellow-green) with nLacZ. (C) nLacZ expression (green) is detected in Lhx3^{+} interneurons (arrowhead; see Figure 1M) nestled among the rhodamine-backlabeled SAC^{d} motor neurons. nLacZ is not detected in SAC^{d} motor neurons. (C, inset) To demonstrate that nLacZ can be expressed from the *CRE-reporter* in SAC^{d} motor neurons, *Pro:CRE* mice were mated to *CRE-reporter* mice. In e13.5 embryos that inherited an activated reporter in all tissues, backlabeled SAC^{d} cells express nLacZ (red-yellow).

(D–F) (D) Hypoglossal (XII^{v}) motor neurons express nLacZ (yellow-green), whereas (E) trigeminal (V^{d}) (red) and (F) trochlear (IV^{d}) (red) do not.

(G–I) (G) LMC^{v} cells that have extended axons to the base of the limb at e10.5 were backlabeled with rhodamine-dextran (red). The backlabeled LMC^{v} cells (red) do not express Lhx3 (green). (H) As Lhx3 cells (green) migrate laterally from the ventricular zone (left to right), they activate the nLacZ reporter (yellow coexpression) in e10.5 F1 embryos. Cells that have migrated laterally to the position of the LMC^{v} (compare G to H) express nLacZ (red) but not Lhx3. (I) Lateral nLacZ cells are motor neurons and coexpress Isl1/2 (red-yellow).

(J) Summary of gene expression during d-MN and v-MN development. (d-MN) Lhx3 and Lhx4 are not detected in mpm2 progenitors of dorsal-exiting motor neuron classes, nor is nLacZ activated by *Lhx3:CRE* in d-MNs. As d-MNs are born, they express Isl1 and later turn on additional LIM-HD genes as their subclass identity is further refined (Ericson et al., 1997). (v-MN) Lhx3 and Lhx4 are first detected in the direct progenitor cells that give rise to Isl1^{+} v-MNs; thus, nLacZ is permanantly expressed in the v-MN lineage in *Lhx3:CRE* mice. A major distinction between d-MN cells and v-MN cells as they are born and extend axons from the neural tube is their Lhx3/4 status. However, as v-MN cells become topographically organized and extend axons in the periphery, MMCm^{v} cells maintain Lhx3/4 expression, whereas the other motor columns rapidly down-regulate Lhx3/4.

Scale bar in (F) corresponds to 32 μm in (A–F) and 80 μm in (G–I).

The apparent difference in Lhx3 expression at early and late stages suggested that most classes of v-MN cells may only transiently express this gene. To examine this, LMC^{v} motor neurons were identified by backlabeling at the earliest stage possible (e10.5) (Figure 4G), and Lhx3, Isl1/2, and nLacZ expression was compared in F1 embryos (Figures 4G–4I). Although motor neurons are still migrating, it is apparent that as Lhx3^{+} cells move laterally from the ventricular zone, a transition occurs in which nLacZ appears in Isl1/2^{+} LMC^{v} cells as Lhx3 disappears (compare Figures 4H and 4I). Our observations show that v-MN cells first begin to express Lhx3 and Lhx4 during their final progenitor cell division (Figures 3A–3C). Once born, they appear to rapidly down-regulate these factors (except the MMCm^{v}) as they become topographically organized into columns and extend axons peripherally (Figure 4J).

An Ectopic Motor Column Forms in Double Knockouts of *Lhx3* and *Lhx4*

Motor columns from C5–L6 are comprised exclusively of v-MN cells that descend from Lhx3^{+} progenitors (see above). Therefore, we examined whether motor neurons are generated at C5–L6 levels in embryos deficient in Lhx3 and Lhx4. In the spinal cord of DKO embryos, we found that the ventral horn was almost completely devoid of Isl1^{+} motor neurons (Figures 5A–5D); instead, a column of Isl1^{+} cells forms along the intermedio-lateral edge of the neural tube from cervical to lumbar levels (Figure 5B; data not shown for each level).

Three criteria were used to define intermedio-lateral Isl1^{+} cells in DKO embryos as motor neurons. First, intermedio-lateral Isl1^{+} cells in DKO embryos are not marked by the interneuron marker Brn3.0 (data not shown), which labels Isl1^{+} interneurons in the dorsal spinal cord (Fedtsova and Turner, 1997). Second, these Isl1^{+} cells express *Choline acetyltransferase* (*ChAT*, Figures 5C and 5D), the rate-limiting enzyme for motor neuron neurotransmitter synthesis. Third, these Isl1^{+} cells extend axons into the periphery (Figures 5E and 5F), a hallmark of motor neurons. Our results demonstrate that motor neuron development can occur in the absence of *Lhx3* and *Lhx4* function, though the topographic organization of motor neuron cell bodies is dramatically altered.

Lhx3 and Lhx4 Are Required for v-MN Differentiation

To define the role of Lhx3 and Lhx4 in the differentiation of individual motor neuron subtypes, we examined the

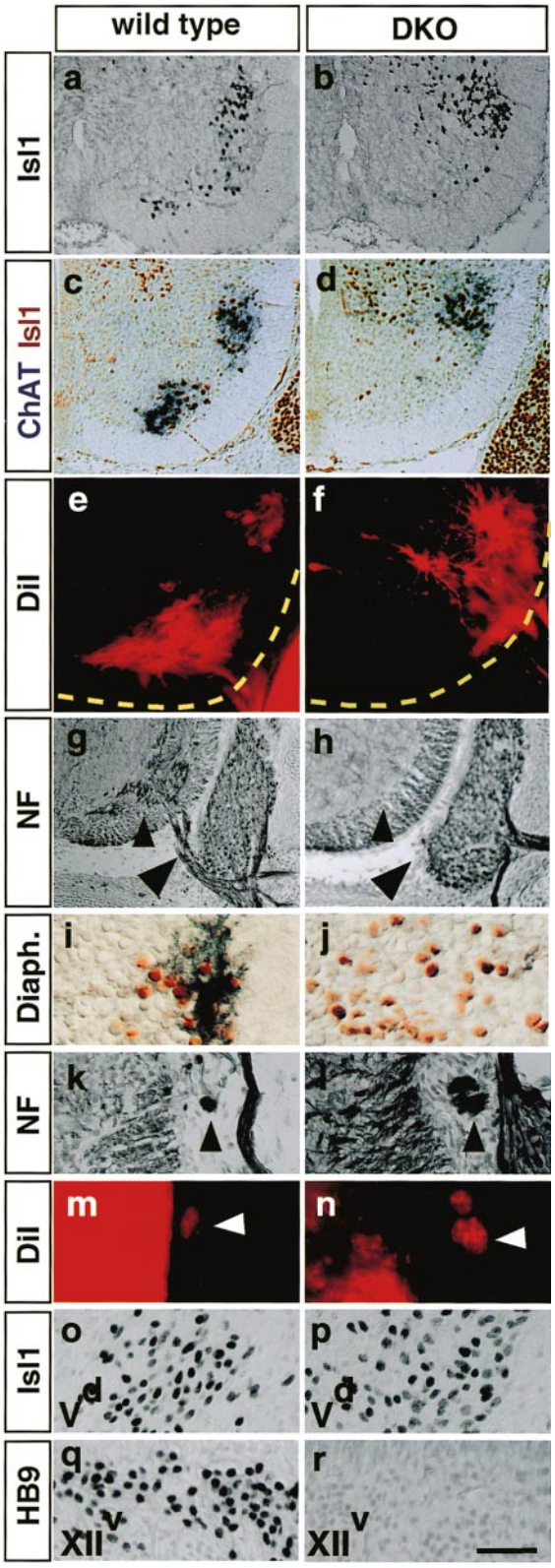

Figure 5. Embryos Deficient in Lhx3 and Lhx4 Lack v-MN Cells but Retain d-MN Cells

(A–B) Isl1$^+$ staining in e13.5 embryos at C6–C8 spinal cord levels. (A) Motor neurons in wild-type embryos are located from ventral–medial to intermedio-lateral positions. (B) Isl1$^+$ cells in DKO embryos are found in intermedio-lateral positions. The number of Isl1$^+$ cells is similar in wild-type and DKO embryos.

(C and D) Immunocytochemical detection of Isl1 (brown) combined with in situ detection of *ChAT* (dark purple). (C) Isl1 motor neurons express *ChAT* in wild-type embryos. (D) Intermedio-lateral Isl1 cells express *ChAT* in DKO embryos. Note that dorsal–medial Isl1 interneurons in (C and D) are *ChAT* negative.

(E and F) Retrograde labeling with DiI. (E) Thoracic peripheral nerve fill in wild-type embryo. (F) SAC^d fill in DKO embryo.

(G and H) Neurofilament staining at thoracic level. (G) Wild-type ventral root and motor neurons (arrowheads). (H) No ventral root is found in serial sections of DKO embryos (arrowhead), and ventral horn motor neurons are not detected (arrowhead).

(I and J) Histochemical detection of NADPH diaphorase activity (Diaph., dark purple) combined with immunocytochemical detection of Isl1 (brown). (I) PGC^v cells in wild-type embryos are double labeled, whereas (J) intermedio-lateral Isl1$^+$ cells in DKO embryos are not.

(K and L) Neurofilament staining at cervical level. (K) Spinal accessory fascicle between the spinal cord and dorsal root ganglia in wild-type embryo (arrowhead). (L) A large spinal accessory fascicle is stained in DKO embryos (arrowhead). Note that (K and L) are the same magnification for comparison.

(M and N) Orthograde DiI labeling of spinal motor axons. (M) Labeling of the spinal accessory fascicle at C2–C4 level in a wild-type embryo (arrowhead). (N) Orthograde fills only label the spinal accessory fascicle in DKO embryos (arrowhead).

(O–R) Immunostaining of hindbrain motor nuclei. Trigeminal motor neurons (V^v) express Isl1 in (O) wild-type and (P) DKO embryos. (Q) Hypoglossal motor neurons (XII^v) express the marker HB9 (Tanabe et al., 1998), whereas (R) no hypoglossal cells are labeled by HB9 in DKO embryos.

Scale bar in (R) corresponds to 100 μm in (A–D), (G), and (H), 80 μm in (E and F), 48 μm in (I), (J), and (O–R), and 32 μm in (K–N).

identity of the Isl1$^+$ motor neurons in DKO embryos. The intermedio-lateral location of motor neuron cell bodies in DKO embryos is similar to SAC^d motor neurons at C1–C4/5, LMC^v motor neurons at C5–C8 and L1–L6, and PGC^v motor neurons at T1–T12 (compare Figures 1H, 1J, 1L, and 5D). Our analysis of marker gene expression suggested that motor neurons in DKO embryos had neither LMC^v nor PGC^v identity. While LMC^v cells normally express Isl2, no Isl2 expression was detected in motor neurons of DKO embryos (data not shown). Many PGC^v motor neurons express NADPH diaphorase activity in their cell bodies (Wetts and Vaughn, 1994); however, NADPH diaphorase activity was not detected in DKO embryos (Figures 5I and 5J).

Neurofilament staining on serial sections and orthograde DiI labeling along the neuraxis revealed that DKO embryos lack ventral roots (Figures 5G and 5H). Therefore, we tested if the Isl1$^+$ motor neurons in DKO embryos have properties of d-MNs, such as the SAC^d. Motor neurons in the SAC^d column are normally located from C1–C4/5 and project axons intermedio-laterally out diffuse rootlets on the side of the cervical spinal cord (see Figure 1). After exiting, their axons form a fascicle between the neural tube and dorsal root ganglia that extends rostrally to a plexus adjacent to the caudal hindbrain (Brichta et al., 1987). In DKO embryos, the SAC^d fascicle is unusually large (Figures 5K and 5L), and the only peripheral motor axons labeled by retrograde or orthograde DiI fills were in the SAC^d nerve (Figures 5F, 5M, and 5N). Thus, motor neurons in the ectopic intermedio-lateral column running the length of the spinal cord in DKO embryos appear to extend axons much

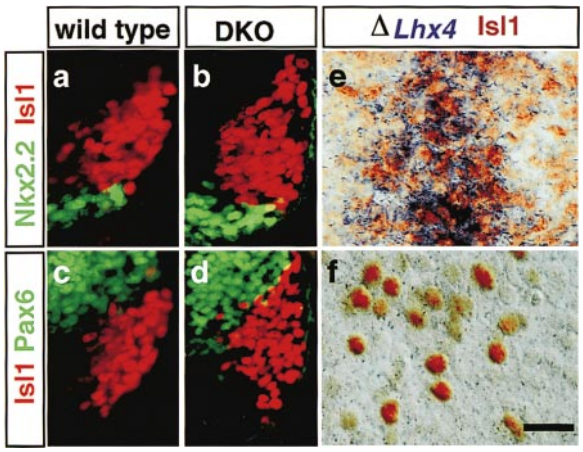

Figure 6. Motor Neuron Generation Appears Normal in DKO Embryos, but They Undergo a Fate Conversion

(A–D) Double label immunofluorescence on the right ventral quadrant of the neural tube in e10.5 embryos. Nkx2.2 (green) defines a ventral region of the neural tube, and Pax6 (green) defines a dorsal area. Isl1$^+$ motor neurons (red) differentiate in the correct dorsal–ventral location in DKO embryos (compare A and B, C and D). Note the number of Isl1$^+$ cells (red) is similar in wild-type and DKO embryos.

(E and F) Immunocytochemical detection of Isl1$^+$ (brown) combined with in situ staining for Δ*Lhx4* (dark purple) in DKO embryos. (E) Isl1 cells express the Δ*Lhx4* transcript as they are generated at e10.5. (F) At e13.5, Isl1$^+$ cells in the intermedio-lateral column no longer have detectable levels of the Δ*Lhx4* transcript.

Scale bar in (F) corresponds to 42 μm in (A–D) and 30 μm in (E and F).

like the SAC^d class of motor neurons at cervical levels in wild-type embryos.

The presence of SAC^d motor neurons and absence of LMC^v, MMCm^v, MMCl^v, and PGC^v motor neurons at spinal levels raised the possibility that Lhx3 and Lhx4 may be specifically required for the differentiation of v-MN cells. We chose to further examine this in the hindbrain, where multiple groups of d-MN and v-MN motor neurons occupy defined rostral–caudal positions. In DKO embryos, no hypoglossal (XII^v) or abducens (VI^v) motor neurons are found (Figures 5Q and 5R; data not shown). In contrast, DKO embryos have d-MN trigeminal (V^d) and trochlear (IV^d) motor neurons (Figures 5O and 5P; data not shown). In summary, we find that v-MN cells fail to differentiate in DKO embryos, whereas the differentiation of both somatic (e.g., IV^d) and visceral (e.g., V^d) d-MN cells persists in embryos lacking Lhx3 and Lhx4 function.

v-MN Fate Conversion in Lhx3- and Lhx4-Deficient Embryos

The loss of v-MN motor neurons accompanied by the appearance of d-MN cells in an intermedio-lateral motor column led us to examine the origin of this phenotype in DKO embryos. First we tested if v-MN cells were eliminated through programmed cell death. In contrast to control experiments with Isl1 knockout embryos, in which motor neurons die (Pfaff et al., 1996), both DKO embryos and wild-type embryos contained few apoptotic cells (data not shown).

Since LIM-HD genes have been implicated in the control of cell fate in invertebrates, we next examined whether d-MN cells in the intermedio-lateral motor column arise from cells expected to become v-MNs. We found that motor neurons are initially generated in the normal dorsal–ventral location relative to cells marked by Pax6 and Nkx2.2 (Figures 6A–6D) (Ericson et al., 1997). In addition, the number of Isl1$^+$ cells per section at e10.5 in DKO embryos (153 ± 8) was approximately normal (179 ± 16). Isl1$^+$ cells in DKO embryos appear to migrate dorsally following their generation, and the ventral horn becomes occupied by Lhx1/5$^+$ (Lim1/2) interneurons (data not shown). At e13.5, intermedio-lateral Isl1$^+$ cells in DKO embryos are found at increased numbers at limb levels (110 ± 20 per section) compared to thoracic levels (59 ± 7 per section), in accordance with the normal variation in motor neuron number along the spinal cord.

To test directly if motor neurons in DKO embryos arise from cells fated to express Lhx3 and Lhx4, we used *Lhx4* RNA as a lineage marker. The knockout of *Lhx4* retains the upstream region of the gene (Li et al., 1994), and a truncated RNA (Δ*Lhx4*) is expressed from this allele. We find that at e10.5, Isl1$^+$ cells express Δ*Lhx4* RNA in DKO embryos (Figure 6E), an indication that these cells arise directly from cells fated to express *Lhx4*. By e13.5, Δ*Lhx4* RNA is no longer detectable in the intermedio-lateral motor column (Figure 6F), an indication that motor neurons acquire an identity that does not maintain Δ*Lhx4* expression.

Misexpression of Lhx3 Reorients Axonal Projections Ventrally

Our analysis of *Lhx3* and *Lhx4* knockouts has shown that these genes are necessary for v-MN differentiation. Therefore, we sought to test whether the Lhx3/4 activity is sufficient to promote ectopic v-MN development. We selected chick embryos for misexpression of Lhx3 for two reasons. Motor neuron development in chick closely parallels that of mouse, and we could temporally and spatially control misexpression in chick (Figure 7A). We electroporated an *Lhx3* expression plasmid in ovo into hindbrain rhombomeres R7–R8 of H.H. stage 11 chick embryos. Normally at R7, d-MN progenitor cells give rise to vagal (X^d) motor neurons, but no v-MN subtypes are generated at this level (Figure 7B). Following the electroporation, embryos were incubated to H.H. stage 24, allowing motor neurons to be born and extend axons from the neural tube. This misexpression paradigm limits ectopic gene expression to one-half of the neural tube, the nonelectroporated side thus serving as an internal control (Figure 7C).

Using neurofilament staining to examine the projections of neurons, we found that an ectopic ventral root forms at R7 on the side of the neural tube in which Lhx3 is ectopically expressed (Figure 7E, left side). This phenotype was never observed with mock electroporations or when LIM-HD genes unrelated to *Lhx3* were electroporated. By stage 24, some of the cell bodies of the vagal motor neurons have begun to translocate dorsally to settle in their final positions (Figure 7D, bracket). In contrast, the number of migrating Isl1$^+$ cells

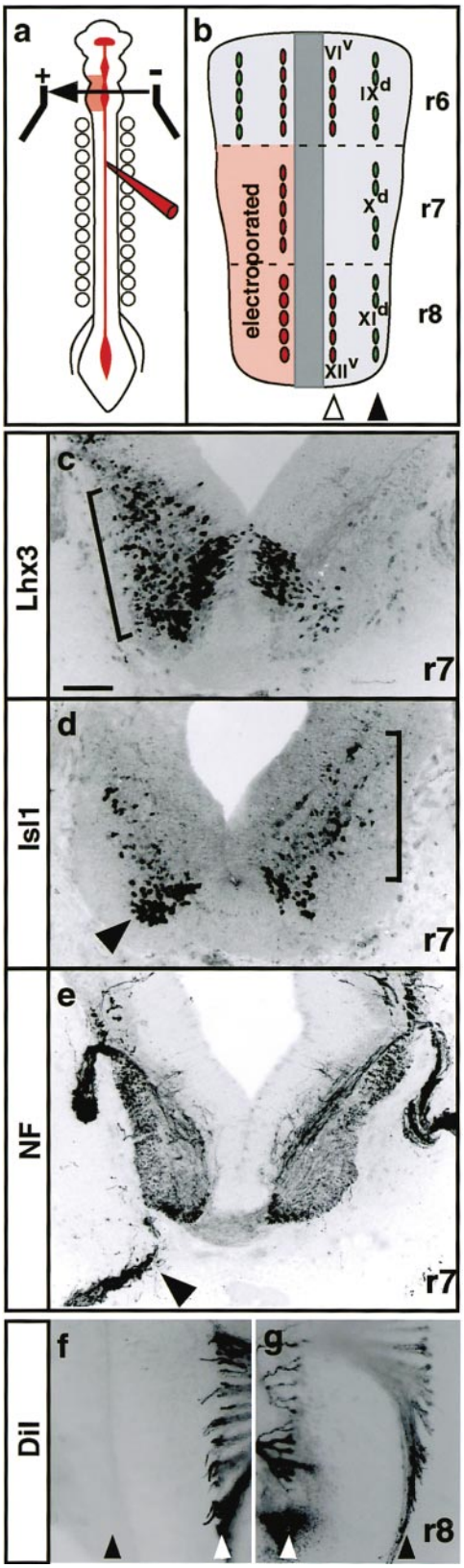

Figure 7. Misexpression of Lhx3 Reorients the Trajectory of Axons from the Neural Tube

(A) Schematic showing the location of *pCS2:Lhx3* DNA injection (red pipette) into the central canal of H.H. stage 11 chick embryos. Progenitor cells for motor neurons are present at this stage, but postmitotic motor neurons have not been generated. Electroporation electrodes (+, −) were placed beside the hindbrain to introduce DNA in ovo into the left side of rhombomeres 7–8.

(B) Schematic of chick H.H. stage 24 rhombomeres (r) 6–8 with dorsal (black arrowhead) and ventral (white arrowhead) motor neuron exit points indicated. Exit points for ventral-exiting motor neurons abducens (VI^v) and hypoglossal (XII^v) in red and dorsal-exiting motor neurons glossopharyngeal (IX^d), vagus (X^d), and cranial accessory (XI^d) in green.

(C–E) Rhombomere 7 at H.H. stage 24 following electroporation. (C) Normal $Lhx3^+$ interneurons form in the ventral hindbrain on the nonelectroporated side (right). The electroporated side (left) has 200–400 ectopic Lhx3 cells per section along the dorsal–ventral axis (bracket). (D) $Isl1^+$ vagal motor neurons (X^d) do not express Lhx3 (right). On the electroporated side (left), many $Isl1^+$ cells express ectopic Lhx3 (arrowhead, compare C and D). Fewer Isl1 cells migrate dorsally on the electroporated side (compare right and left dorsal regions defined by bracket). (E) Neurofilament (NF) staining reveals an ectopic ventral root on the electroportated side of the neural tube (arrowhead). Neurofilament also labels the dorsal root comprised of both motor and sensory fibers.

(F and G) Orthograde DiI labeling of efferent projections at rhombomere 8 in efficiently electroporated H.H. stage 24 chicks. After axonal labeling, the neural tube was removed to reveal the motor axon exit points in an open-book preparation of the peripheral tissue. (F) On the electroporated (left) side, hypoglossal (XII^v) ventral roots are labeled (white arrowhead), but dorsal roots from the spinal accessory motor neurons (XI^d) are not detected (black arrowhead). (G) Nonelectroporated (right) side of same embryo as (F), showing ventral roots for the hypoglossal (XII^v) (white arrowhead) and dorsal roots from the spinal accessory motor neurons (XI^d) (black arrowhead). The schematic in (B) summarizes these results on the left side of r8.

Scale bar in (C) is 110 μm in (C–E) and 60 μm in (F and G).

is reduced on the left side of R7 in which Lhx3 is misexpressed (Figure 7D). While Lhx3 misexpression leads to changes in motor neuron cell body position and axonal projections, Lhx3 does not appear to change the number of $Isl1^+$ motor neurons that form. Sections of R7 with >300 ectopic Lhx3 cells have 84 $Isl1^+$ motor neurons, compared to 79 on the control side. These results are consistent with the possibility that v-MN cells have been generated at the expense of d-MN cells by ectopically expressing Lhx3. This is supported by our findings that very efficient electroporation of *Lhx3* into the R8 level, containing both the hypoglossal (XII^v) and cranial accessory (XI^d) motor neurons, selectively suppresses the appearance of d-MNs on the electroporated side of the neural tube (compare Figures 7F and 7G).

Discussion

As motor neurons begin to extend axons during embryonic development, two distinct subtypes can be identified. The axons of one subtype emerge ventrally from the neural tube (v-MN), while axons of the other subtype emerge from dorsally located exit points (d-MN). In this study we provide evidence that *Lhx3* and *Lhx4* specify v-MNs. Axonal exit point identity appears to be determined during a brief period in motor neuron development, as Lhx3 and Lhx4 are only expressed transiently in most classes of v-MNs. We discuss the implications of these findings for the specification of motor neuron

identity and the role of LIM-HD genes in establishing appropriate motor connectivity.

Generation of Motor Neuron Diversity

Our study reveals an important genetic distinction between motor neuron subtypes that can be categorized as v-MN or d-MN. Notably, embryos deficient in both Lhx3 and Lhx4 lack v-MN cells but retain d-MNs. It is likely that Lhx3 and Lhx4 are directly required for the differentiation of v-MNs, as CRE-mediated lineage tracing revealed that all classes of hindbrain v-MN motor nuclei and all spinal v-MN motor columns contained lineage-marked cells (Figure 4J). In contrast, d-MN motor neurons were never marked by CRE. Because Lhx3 and Lhx4 are not required for motor neuron development, even at C5–L6 spinal cord levels in which only v-MN motor neurons differentiate, it is likely that a generic program for motor neuron differentiation operates independently from the activities of these LIM-HD factors.

Previously, it was unclear whether somatic and visceral identity were coordinately specified along with exit point identity. These properties appear to be linked, as somatic motor neurons project ventrally from the spinal cord and visceral motor neurons project dorsally from the hindbrain. Our finding that Lhx3 and Lhx4 are required for visceral PGC^{v}, but not somatic trochlear (IV^{d}), motor neurons provides evidence that v-MN/d-MN identity is acquired separately from somatic/visceral identity (Ericson et al., 1997; Tanabe et al., 1998).

One of the clearest distinctions among motor neuron subtypes is the initial axonal trajectory they take to exit the neural tube (Lumsden and Krumlauf, 1996). Motor neuron axon exit points appear to have changed significantly in the course of vertebrate evolution. In primitive chordates such as amphioxus, motor neurons exit the spinal cord by projecting axons dorsally from the neuroepithelium along with Rohon Beard sensory neurons (Fritzsch and Northcutt, 1993). With the appearance of vertebrates such as lamprey, dorsal-exiting motor neurons have been replaced by ventral-exiting cells in the spinal cord. However, in the hindbrain of vertebrates many of the cranial motor nuclei retain the ancestral motor neuron projection pattern and still extend axons dorsally from the neural tube (Fritzsch and Northcutt, 1993). Our data raise the possibility that *Lhx3* and *Lhx4* have contributed to the derivation of v-MN cells in vertebrates.

v-MN Cells Convert into d-MN Cells in DKO Embryos

Embryos deficient in both Lhx3 and Lhx4 appear to initially generate motor neurons at the correct dorsal–ventral position, in normal numbers, on a normal developmental schedule. However, several lines of evidence suggest that motor neurons acquire inappropriate identities in DKO embryos. First, the expression of LIM-HD factors in combinatorial patterns in motor neuron subtypes does not occur normally (data not shown). Second, motor neurons of the v-MN subtype normally settle into stereotyped locations in the ventral spinal cord, where they receive specific presynaptic input. In DKO embryos, motor neurons settle in an unusual intermedio-lateral column within the neural tube, which is analogous to the dorsal positions of many of the d-MN cranial motor nuclei and spinal accessory column. Third, motor neurons fail to project axons into the ventral root in DKO embryos; instead, they appear to exit the neural tube from intermedio-lateral locations. Our analysis of LIM-HD gene expression, cell body position, and axon projections clearly demonstrate that motor neurons have d-MN properties in embryos devoid of Lhx3 and Lhx4.

What is the fate of cells that would normally be expected to acquire v-MN cell identity? By tracing the truncated *Lhx4* transcript to early Isl1^{+} cells, we show that v-MN cells directly convert into cells that eventually settle in the intermedio-lateral motor column of DKOs. The apparent fate conversion between v-MN and d-MN cells raises the possibility that the progenitors for these two classes of motor neurons are related and differ primarily in their expression of Lhx3 and Lhx4 (Figure 4J). This is supported by our finding that the misexpression of Lhx3 in d-MN progenitors at R7–R8 led to the generation of motor neurons with v-MN axonal projections at the expense of d-MN formation. Together, our results indicate that a binary choice is operative in motor neuron progenitor cells in which motor neuron axonal exit point identity is defined by the Lhx3 and Lhx4 status of these cells as they give rise to postmitotic motor neuron daughter cells.

The LIM-HD Gene Code and Axon Pathfinding

Studies in *C. elegans* and *D. melanogaster* (Lundgren et al., 1995; Thor and Thomas, 1997; Hobert et al., 1998) suggest that LIM-HD factors regulate the expression of target genes for neuronal pathfinding. In addition, in vitro studies have begun to reveal differences in the response of d-MN and v-MN cells to cues that may control their axonal projections from the neural tube (Colamarino and Tessier-Lavigne, 1995; Guthrie and Pini, 1995; Varela-Echavarria et al., 1997). Thus, it is likely that Lhx3 and Lhx4 contribute to the regulation of the expression of receptors for ventral axonal projections from the neural tube. In Lhx3- and Lhx4-deficient embryos, the default program of gene expression for axon guidance appears to mimic that of d-MN cells.

Why are Lhx3 and Lhx4 rapidly extinguished from all v-MN classes except the MMCm^{v} (see Figure 4J)? As originally proposed for LIM-HD genes (Tsuchida et al., 1994), it is possible that Lhx3 and Lhx4 also contribute to the regulation of genes for motor neuron axon pathfinding in the periphery of the embryo. Our studies of *Lhx3* and *Lhx4* knockouts were not able to reveal this function because of the cell fate conversion to a d-MN identity. To define the later functions of these LIM-HD genes in axon guidance, it may be useful to artificially maintain Lhx3 and Lhx4 expression in v-MN cells that would normally down-regulate these factors during the later period in which they extend axons peripherally.

v-MN Subtype Identity Is Determined at the Time Motor Neurons Are Specified

Motor neuron subtype identity appears to be specified at several distinct points in development (Lance-Jones

and Landmesser, 1980; Matise and Lance-Jones, 1996; Sockanathan and Jessell, 1998). Studies of Shh have defined two critical steps for the specification of generic motor neuron identity. These signaling events operate in dividing neuroepithelial cells, committing progenitor cells to a motor neuron fate during their final cell division (Leber et al., 1990; Ericson et al., 1996). In this study we provide evidence that Lhx3 and Lhx4 are expressed in dividing cells by showing colocalization of these factors with mitotic cell markers. Thus, the v-MN lineage is derived primarily from cells that transiently express Lhx3 and Lhx4 during the progenitor cell phase in which motor neuron identity is thought to be established by Shh. The brief expression of Lhx3 and Lhx4 in v-MN progenitor cells suggests that additional factors controlling critical aspects of neuronal identity in other regions of the nervous system may also act during a single cell cycle.

Experimental Procedures

Immunocytochemistry and In Situ Hybridization

Amino acids 266–402 of mouse Lhx3 (Zhadanov et al., 1995) and amino acids 93–224 of Lhx4 (Li et al., 1994) were cloned into pGEX (Pharmacia) for expression in *E. coli* and purification on glutathione columns. These antigens were used to immunize rabbits. Immunocytochemistry was performed as described previously (Pfaff et al., 1996), using the following dilutions: rabbit anti-Lhx3 serum, 1:5,000; rabbit anti-Lhx4 serum, 1:4,000; rabbit anti-Isl1/2 serum, 1:5,000 (Karlsson et al., 1990); rabbit anti-Isl1 serum, 1:8,000 (Tsuchida et al., 1994); mouse monoclonal anti-Pax6, 1:100 (Ericson et al., 1997); mouse monoclonal anti-Nkx2.2, 1:100 (Ericson et al., 1997); mpm2, 1:10,000 (Upstate Biotechnology Incorporated) (Westendorf et al., 1994); rabbit anti-LacZ, 1:10,000 (Sigma); and rabbit anti-neurofilament, 1:10,000 (Cappel). Species-specific secondary antibodies were conjugated to FITC or Cy3 and used as recommended (Jackson Labs). Images were obtained with a Zeiss Axioplan II microscope and a Princeton Instrument MicroMax cooled CCD camera and electronically assigned to red or green channels.

Digoxigenin-labeled riboprobes complementary to Δ*Lhx4* and *ChAT* were synthesized according to supplier protocol (Boehringer Mannheim) and used for in situ hybridization as described previously (Schaeren-Wiemars and Gerfin-Moser, 1993). NADPH diaphorase staining was performed on cryosectioned material (Wetts and Vaughn, 1994).

Neuronal Fills

Backlabeling of specific motor neuron subtypes was performed with 3000 MW rhodamine-dextran (Molecular Probes). Embryos were cultured in oxygenated mouse Ringer's solution for 6–10 hr at room temperature to permit retrograde transport of the label and then fixed for immunocytochemistry. Retrograde and orthograde labeling with DiI was performed by injection of label in ethanol as described previously (Sharma et al., 1994). Following DiI injection, embryos were incubated in 4% paraformaldehyde for 1 week at 37°C to permit diffusion. Embryos were vibratome sectioned at 50–75 mm for analysis.

Mice

An oligonucleotide with a PacI site was inserted into the HindIII site located 735 nucleotides downstream of the end of translation of a 4.4 kb genomic clone of *Lhx3*. A 3.6 kb *IRES-CRE-NEO* cassette was cloned into the PacI site created in the *Lhx3* genomic clone and electroporated into W95 embryonic stem cells (Pfaff et al., 1996). Homologous recombinants were identified by digesting genomic DNA with EcoRI and probing with a 5′ NotI fragment from the *Lhx3* cDNA *pcdc8*. Chimeric mice, which transmitted the targeted allele, were generated by standard methods (Hogan et al., 1994). Targeted mutations in *Lhx3* and *Lhx4* have been described previously (Li et al., 1994; Sheng et al., 1997). *Lhx3* and *Lhx4* knockout mice were intercrossed to generate embryos lacking both *Lhx3* and *Lhx4*. CRE-reporter mice have been described previously (Tsien et al., 1996).

Lhx3 In Ovo Electroporation

Lhx3 from cDNA clone *pcdc8* (Zhadanov et al., 1995) was inserted into the CMV-based expression vector *pCS2* (D. Turner and H. Weintraub, unpublished). H.H. stage 11 chick embryos were windowed, DNA (5 mg/ml in TE) was pipetted into the lumen of the neural tube, and electrodes were placed on either side of the hindbrain over the vitelline membrane. A square wave electroporator (BTX) was used to administer five pulses of current at 25 volts for durations of 50 ms each. Eggs were sealed, and the embryos were allowed to develop to H.H. stage 24 and prepared for immunocytochemical analysis.

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