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Mesodermal and neuronal retinoids regulate the induction and maintenance of limb innervating spinal motor neurons

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Abstract

During embryonic development, the generation, diversification and maintenance of spinal motor neurons depend upon extrinsic signals that are tightly regulated. Retinoic acid (RA) is necessary for specifying the fates of forelimb-innervating motor neurons of the Lateral Motor Column (LMC), and the specification of LMC neurons into medial and lateral subtypes. Previous studies implicate motor neurons as the relevant source of RA for specifying lateral LMC fates at forelimb levels. However, at the time of LMC diversification, a significant amount of retinoids in the spinal cord originates from the adjacent paraxial mesoderm. Here we employ mouse genetics to show that RA derived from the paraxial mesoderm is required for lateral LMC induction at forelimb and hindlimb levels, demonstrating that mesodermally synthesized RA functions as a second source of signals to specify lateral LMC identity. Furthermore, reduced RA levels in postmitotic motor neurons result in a decrease of medial and lateral LMC neurons, and abnormal axonal projections in the limb; invoking additional roles for neuronally synthesized RA in motor neuron maintenance and survival. These findings suggest that during embryogenesis, mesodermal and neuronal retinoids act coordinately to establish and maintain appropriate cohorts of spinal motor neurons that innervate target muscles in the limb.
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Introduction

The generation and maintenance of different neuronal subtypes during embryonic development are essential events in the establishment of a functional nervous system. In vertebrates, this complex process involves the implementation of cell-intrinsic events by extrinsic signals emanating from local cellular sources (Jessell and Melton, 1992; Liu and Joyner, 2001). In many cases, inductive signals are expressed both by cells adjacent to the nervous system, and by cells resident within the nervous system itself (Jessell, 2000). However, few studies have examined the relative contributions of these different sources towards specifying neuronal fates, or if there are later requirements for these signals in maintaining neuronal numbers.

One model system where the expression of extrinsic factors known to be involved in neuronal fate specification is juxtaposed in adjacent tissues is in developing spinal motor neurons (Jessell, 2000). All motor neurons derive from a distinct ventral progenitor domain, set up in part by *sonic hedgehog* secreted by the notochord and floorplate (Jessell, 2000; Shirasaki and Pfaff, 2002; Price and Briscoe, 2004). The cell bodies of motor neurons are organized into distinct columns defined by their position along the rostral–caudal axis and their stereotypic axonal projections in the periphery (Landmesser, 1978; Hollyday, 1980a,b; Gutman et al., 1993). Motor neurons of the medial Median Motor Column (MMC) are found at all axial levels whereas lateral MMC neurons and preganglionic

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autonomic motor neurons are located specifically in thoracic regions (Prasad and Hollyday, 1991; Gutman et al., 1993; Cornbrooks et al., 1997). Motor neurons of the Lateral Motor Column (LMC) are generated exclusively at forelimb and hindlimb levels and form medial (LMCm) and lateral (LMCl) divisional subtypes which project to ventral and dorsal muscles in the limb, respectively (Landmesser, 1978; Hollyday, 1980a,b; Tosney and Landmesser, 1985a,b). Individual motor columns and divisions can be molecularly defined by the combinatorial expression of LIM homeodomain protein family members (Tsuchida et al., 1994). These transcription factors functionally contribute to the settling patterns of motor neuron cell bodies and their axonal projection profiles (Pfaff et al., 1996; Sharma et al., 1998, 2000; Kania et al., 2000).

Biochemical studies, combined with analyses of reporter mice sensitive to activated retinoid receptors, demonstrate that retinoids are present within the developing spinal cord at the time of motor neuron generation and specification; suggesting a requirement for RA signaling during these events (Rossant et al., 1991; McCaffery and Drager, 1994; Solomin et al., 1998). Recently, work in chicks and Vitamin A deficient quails describes critical roles for RA signaling in establishing and generating motor neuron progenitors, and in mediating their subsequent differentiation into postmitotic motor neurons (Novitch et al., 2003; Diez del Corral et al., 2003; Wilson et al., 2004). Remarkably, retinoids can also influence the fates of motor neurons postmitotically. When dominant-negative retinoid receptors are introduced into postmitotic spinal motor neurons, forelimb level motor neurons do not acquire LMC subtype identities but instead adopt thoracic motor column properties (Sockanathan et al., 2003). This suggests that retinoid signaling imposes forelimb LMC identities partly through the suppression of thoracic fates. Surprisingly, hindlimb LMC formation is not perturbed, suggesting that lumbar LMC identity may be specified by alternative mechanisms (Sockanathan et al., 2003). In addition to influencing columnar identity, retinoids are also required for the divisional specification of LMC neurons. Experiments in the chick show that retinoids can induce LMCl neurons and thus contribute to the diversification of LMC motor neurons into medial and lateral subtypes. Further, retinoid signals are also required for the maintenance of LMCl properties during the migration of these neurons to their final settling position (Sockanathan and Jessell, 1998).

What is the source of retinoids responsible for these critical events in motor neuron specification and maintenance? Biochemical, gene expression and genetic studies have shown that retinaldehyde dehydrogenase 2 (RALDH2) is the major retinoid synthetic enzyme present during embryonic development (Zhao et al., 1996; Niederreither et al., 1997, 1999; Swindell et al., 1999; Mic et al., 2002). RALDH2 is found in the meninges surrounding the neural tube, and is dynamically expressed within the paraxial mesoderm directly adjacent to the spinal cord. Initially, paraxial mesoderm-specific expression of RALDH2 is found at all axial levels; however, higher levels of expression are subsequently concentrated at forelimb regions (Niederreither et al., 1997; Berggren et al., 1999; Swindell et al., 1999; Blentic et al., 2003). At later stages of development, RALDH2 is detected within the spinal cord and is localized to LMC motor neurons (Zhao et al., 1996; Sockanathan and Jessell, 1998). Studies of *Raldh2* null embryos rescued from early lethality by maternally administered RA confirm that mesodermal and neuronal RALDH2 expression are primarily responsible for retinoid levels within the spinal cord (Mic et al., 2002; Niederreither et al., 2002). Given that RALDH2 expression prior to LMC generation is largely mesodermal, both motor neuron generation and LMC formation are likely dependent on paraxial mesoderm-derived retinoid signaling. In contrast, the specification and maintenance of LMCl neurons are believed to rely on retinoids generated by early born LMCm neurons. However, at the time of LMC diversification into medial and lateral subtypes, retinoids in the spinal cord derive from both paraxial mesoderm and motor neuron sites of RALDH2 expression (Niederreither et al., 1997, 2002; Berggren et al., 1999; Swindell et al., 1999; Mic et al., 2002; Blentic et al., 2003).

Attempts to define the specific contributions of each retinoid source to LMC subtype specification and LMC maintenance have been stymied by the early lethality of *Raldh2* null embryos prior to motor neuron generation (Niederreither et al., 1999). A recent study where *Raldh2* expression was genetically ablated in forelimb spinal motor neurons led to a decrease of forelimb LMCl neurons, supporting the idea that motor neuron sources of RA are partly required for LMCl specification (Vermot et al., 2005, Fig. 8A). The homeobox protein Hoxc8 is thought to mediate this process as Hoxc8 expression is lost in LMC neurons in mice lacking motor neuron-specific expression of RALDH2, and there are reduced numbers, and aberrant axonal projections of remaining LMCl cells in Hoxc8 mutants (Vermot et al., 2005). However, one striking observation from these studies is that 80% of LMCl neurons are preserved in the absence of motor neuron sources of RA. Given that significant amounts of RA within the spinal cord derive from RALDH2 activity in the adjacent paraxial mesoderm, it is possible that RA synthesized in the paraxial mesoderm provides an important source of retinoids for specifying LMCl identity (Mic et al., 2002; Niederreither et al., 2002). Moreover, the contribution of mesodermal and neuronally synthesized retinoids to hindlimb LMC development and maintenance of LMC neurons respectively remains unknown.

Here we have used genetic approaches to independently reduce RALDH2 levels in paraxial mesoderm and in spinal motor neurons at forelimb and hindlimb levels of the spinal cord to examine the requirements for mesodermal and neuronal retinoids in LMC specification and maintenance. We find that reduction of RALDH2 in the paraxial mesoderm results in the specific decrease of both forelimb and hindlimb LMCl neurons, whereas LMCm neurons are unaffected. Thus, RA derived from the mesoderm functions as an additional source of signals to specify LMCl fates (Fig. 8B). In contrast, knockdown of RALDH2 in postmitotic motor neurons results in reduced numbers of forelimb and hindlimb LMCm and LMCl neurons later in development, and the atrophy of remaining limb-innervating neurons. This result suggests that continued RA

synthesis in motor neurons may be critical for maintenance of LMC neuronal numbers and properties (Fig. 8C). Given that the majority of remaining forelimb LMC neurons lose Hoxc8 expression, we suggest that Hox proteins may partly mediate the retinoid-dependent maintenance of LMC neurons. Taken together, these genetic studies uncover new roles for mesodermal and neuronal retinoids in the specification, maintenance and survival of spinal motor neurons that target the limb.

Materials and methods

Generation of mouse lines

Raldh2^{lox} line: Mouse genomic clones were isolated from a 129/Sv genomic library (Stratagene) and loxP sites introduced into the *Nsi*I and *Kpn*I sites located 5′ and 3′ of Exon 4. Introduction of a thymidine kinase promoter neomycin/hygromycin cassette into the *Kpn*I site in the reverse direction resulted in an additional *Eco*RI site within the *Raldh2* locus, generating an 11 kb *Eco*RI band when homologously recombined instead of the wild-type 14 kb band. A 2 kb *Kpn*I fragment outside the targeting construct was used as a 3′ probe. ES cell transfections, screening and injection into blastocysts were carried out according to standard procedures. *Raldh2$^{+/-}$* lines were generated by integrating a pgk neomycin cassette within the *Cla*I site of Exon 4. Details for *msd:Cre* mice are available by request from A. Gossler. Specific primers and PCR protocols used to genotype *Cre*, *Raldh2$^{+/-}$* and *Raldh2^{lox}* alleles are available upon request. *Gtrosa26^{tm1Sor}* mice were obtained from Jackson Laboratories. *RARE-hspLacZ* mice engineered by Rossant et al. (1991) were obtained from C. Mendelsohn with the permission of J. Rossant.

Production of RALDH2 antibodies

A C-terminal mouse RALDH2 peptide (SEVKTVTVKIPQKNS) was coupled to keyhole limpet hemocyanin and injected into guinea pigs (Covance). Sera were affinity purified using a peptide column (ABR).

In situ hybridization and immunohistochemistry

In situ hybridization was performed as described by Schaeren-Wiemers and Gerfin-Moser (1993). The mouse *Raldh2* in situ probe consisted of a 937 bp fragment from the ATG. Immunohistochemistry on sections and whole mounts was performed as described in Sockanathan and Jessell (1998) and Giger et al. (2000). Images were acquired using Zeiss LSM 510 or LSM 5 PASCAL confocal microscopes. Primary antibodies were used at the following dilutions: K5 (rabbit anti-Isl1/2), 1:3000; guinea pig anti-Isl1/2, 1:20000; rabbit anti-Pea3, 1:1000 (Arber et al., 1999); rabbit anti-Lhx3, 1:3000 (Sharma et al., 1998); 4F2 (mouse anti-Lim1/2) 1:2, mouse anti-Hoxc8 1:200 (all provided by T.M. Jessell) and 2H3 (anti-neurofilament) 1:50 (Developmental Studies Hybridoma Bank, University of Iowa); guinea pig anti-RALDH2, 1:20000; rabbit anti-RALDH2, 1:2000 (provided by P. McCaffery); rabbit anti-LacZ, 1:1000 (Cappel, Durham, NC). Beta-galactosidase staining on tissue sections was carried out using standard protocols.

Motor neuron counts

Motor neuron counts were confined to the limb regions corresponding to the entire rostrocaudal extent of the LMC, identified by Isl2^{+}/Lim1^{+} neurons. Neurons in approximately 15–18 sections/embryo were counted, depending on developmental stage. Typically 3–6 embryos were analyzed for each experiment.

Western blots

Western blots were performed according to standard protocols and bands were visualized using ECL Plus™ Western Blotting Detection Reagents (GE Healthcare). Densitometry analysis was carried out using ImageJ (NIH). Rabbit anti-RALDH2 antibodies were used at a 1:5000 dilution.

RA bioassay

The RA reporter cell line F9-RARE-lacZ (Sil-15) was maintained according to standard protocols (Wagner et al., 1992). Tissue preparation and reporter assays were carried out as described by Haselbeck et al. (1997).

Results

Generation of Raldh2 conditional knockout mice

To examine the contribution of mesodermal and neuronal retinoids to LMC specification, we used Cre-lox technology (Rajewsky et al., 1996) to specifically ablate RALDH2 in either the paraxial mesoderm or in spinal motor neurons. Fig. S1 shows the targeting strategy used to introduce lox-P sites flanking exon 4 of one *Raldh2* allele in the mouse genome (*Raldh2^{lox}*). Exon 4 was chosen as it encodes the nicotinamide adenine dinucleotide (NAD) binding domain essential for RALDH2 activity; removal of this exon results in a non-functional RALDH2 enzyme (Niederreither et al., 1999; Vermot et al., 2003, 2005). Homozygous *Raldh2$^{lox/lox}$* mice were crossed to mice that contained a single *Raldh2* allele inactivated by insertion of the neomycin gene within exon 4 to generate *Raldh2$^{lox/-}$* lines (*Raldh2$^{/-}$*, Fig. S1). *Raldh2$^{lox/-}$* mice were viable, fertile and morphologically indistinguishable from heterozygote littermates, in contrast to *Raldh2$^{-/-}$* mice which die at embryonic day *post-coitum* 9 (E9).

Targeted loss of Raldh2 in the paraxial mesoderm

In order to generate embryos with reduced RALDH2 in the paraxial mesoderm, we utilized transgenic mice that expressed Cre recombinase under the control of the "*msd*" fragment from the Delta1 promoter (*msd:Cre*), which drives heterologous gene expression in the paraxial mesoderm from E7 (Beckers et al., 2000). We first monitored the profile of Cre recombinase activity in *msd:Cre* mice by breeding them with mouse reporter lines that express betagalactosidase upon Cre-mediated recombination (*Gtrosa26^{tm1Sor}*; Soriano, 1999). Strong betagalactosidase staining is detected in forelimb level paraxial mesoderm at E9.5 of *msd:Cre^{+} Gtrosa26^{tm1Sor}* embryos prior to spinal motor neuron generation, and is maintained until at least E12.5 (Figs. 1A, E, Nornes and Carry, 1978). At these developmental stages, betagalactosidase activity within the spinal cord was localized exclusively within the vasculature. Thus, *msd:Cre^{+} RALDH2$^{lox/-}$* embryos should lack RALDH2 in the paraxial mesoderm but preserve LMC-specific expression of the enzyme.

Msd:Cre^{+} Raldh2$^{lox/-}$ embryos are morphologically normal, and survive to birth and adulthood. Embryos were examined at E10.0 and at E12.5 to determine the extent of RALDH2 protein loss in the paraxial mesoderm at forelimb levels compared with *Raldh2$^{lox/-}$* littermates. Forelimb embryonic regions were serially sectioned and an antibody specific to RALDH2 was used to detect the protein by indirect immunofluorescence. Mesodermal RALDH2 expression was significantly diminished in *msd:Cre^{+} Raldh2$^{lox/-}$* embryos compared with *Raldh2$^{lox/-}$* littermates at E10.0, a developmental time-point when

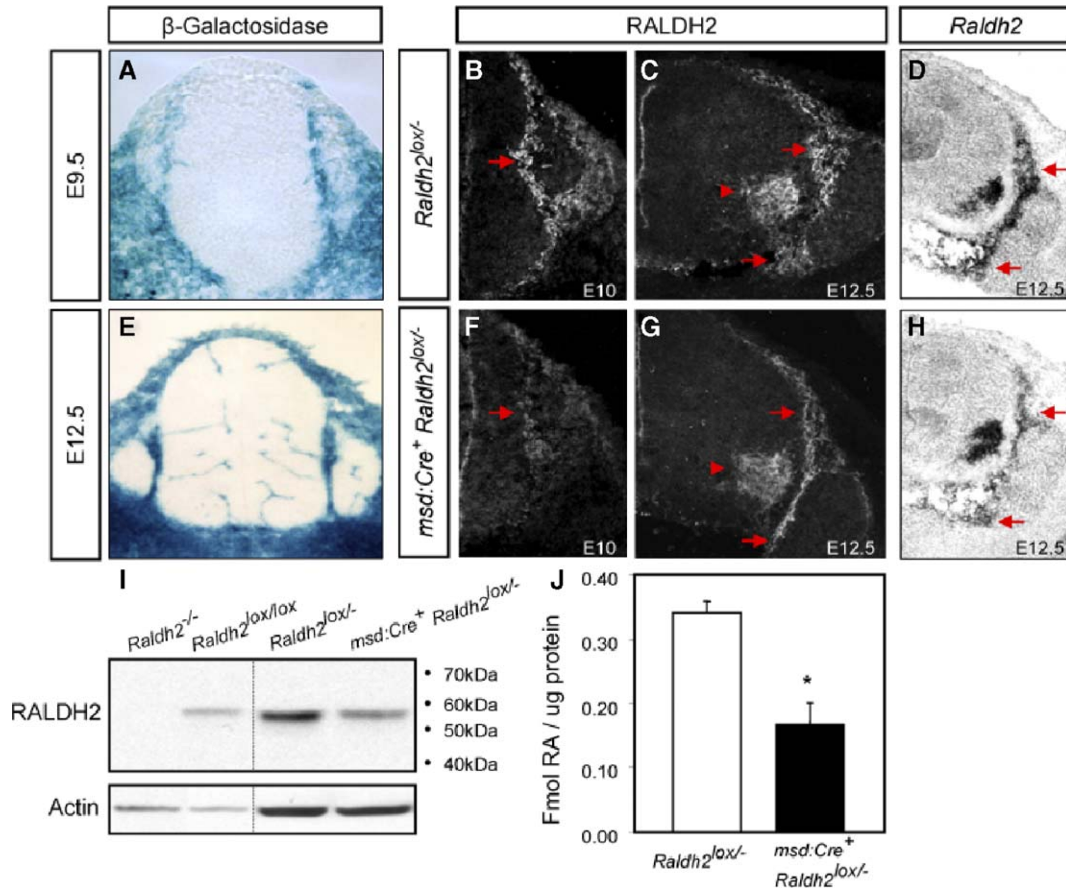

Fig. 1. RALDH2 and RA are decreased in paraxial mesoderm and spinal cords of *msd:Cre*$^+$ *RALDH2*$^{lox/-}$ mutants. (A, E) Betagalactosidase expression of *msd:Cre*$^+$ *Gtrosa26*^{tm1Sor} embryos at E9.5 and E12.5 showing Cre recombinase activity within the paraxial mesoderm but not within motor neurons of the spinal cord. (B, C, E, F) Confocal micrographs of right spinal cord halves, showing RALDH2 expression at E10 and E12.5. Arrows mark decreased RALDH2 expression in paraxial mesoderm. Arrowheads mark preserved RALDH2 expression in LMC neurons. (D, H) In situ hybridization of *Raldh2* mRNA. Arrows mark decreased *Raldh2* expression in *msd:Cre*$^+$ *RALDH2*$^{lox/-}$ mutants compared with controls (I) Western blot analysis of RALDH2 in embryonic extracts. Actin staining is used as a control for ensuring that equivalent amounts of protein were loaded per lane. Extracts prepared from E9 embryos show absence of a 55 kDa band corresponding to RALDH2 in *Raldh2*$^{-/-}$ embryos compared with *Raldh2*$^{lox/lox}$ littermates. E9 embryos were used due to early lethality of *Raldh2* nulls between E9 and E9.5. RALDH2 is decreased by 50% in *msd:Cre*$^+$ *Raldh2*$^{lox/-}$ embryos compared with *Raldh2*$^{lox/-}$ controls at E10. (J) Graph depicting the amount of RA produced in spinal cords of *Raldh2*$^{lox/-}$ and *msd:Cre*$^+$ *Raldh2*$^{lox/-}$ E12.5 embryos (mean ± SEM, Student's *t* test, $*P = 0.001$, $n = 8$ mutants, 4 controls).

RALDH2 is not expressed in the spinal cord (Figs. 1B, F). This decrease was quantified by densitometric analysis of Western blots using protein extracts from embryos dissected at E10. A 55 kDa band corresponding to RALDH2 was reduced by approximately 50% in extracts of *msd:Cre*$^+$ *Raldh2*$^{lox/-}$ embryos compared with *Raldh2*$^{lox/-}$ controls (Fig. 1I). At E12.5, *msd:Cre*$^+$ *Raldh2*$^{lox/-}$ embryos showed decreased *Raldh2* mRNA and protein expression in the paraxial mesoderm relative to controls; however, no differences were detected in the expression of RALDH2 in LMC neurons (Figs. 1C, D, G, H). Together, these results indicate that *msd:Cre*$^+$ *Raldh2*$^{lox/-}$ embryos have diminished levels of RALDH2 expression in the paraxial mesoderm at forelimb levels, but preserve expression of RALDH2 in LMC motor neurons.

We next examined if reduced RALDH2 expression in the paraxial mesoderm led to a measurable drop in RA levels within the spinal cord. Forelimb level spinal cords were dissected from E12.5 *msd:Cre*$^+$ *Raldh2*$^{lox/-}$ embryos and *Raldh2*$^{lox/-}$ littermates, and analyzed using the F9-RARE-LacZ reporter cell line bioassay for measuring RA levels in embryonic tissues (Wagner et al., 1992; McCaffery and Drager, 1994). Tissue homogenates derived from forelimb regions of *msd:Cre*$^+$ *Raldh2*$^{lox/-}$ embryos showed a 50% reduction in RA levels compared with those prepared from *Raldh2*$^{lox/-}$ littermates (Fig. 1J). The remaining 50% of RA in these spinal cords is attributed to preserved RALDH2 expression in LMC neurons, residual retinoids derived from remaining RALDH2 in the paraxial mesoderm and an RALDH2-independent source found in the intermediate (i) region of the spinal cord (Mic et al., 2002; Niederreither et al., 2002). There was no detectable difference in the levels of the i-region RA source between *msd:Cre*$^+$ *Raldh2*$^{lox/-}$ embryos and controls (Fig. S2). Thus, the reduced RA levels in forelimb level spinal cords of *msd:Cre*$^+$ *Raldh2*$^{lox/-}$ embryos can be attributed to decreased RA synthesis in the adjacent paraxial mesoderm.

RA in the paraxial mesoderm is required for forelimb and hindlimb LMCl induction

Previous studies in the chick have shown that paraxial mesoderm-derived retinoids are required for generic motor

neuron generation (Diez del Corral et al., 2003; Novitch et al., 2003) and the specification of forelimb LMC neuronal identity (Sockanathan et al., 2003), both of which occur prior to the diversification of medial and lateral LMC neurons. We examined if either of these events was compromised in *msd:Cre*$^+$ *Raldh2*$^{lox/-}$ embryos. We first quantified the number of Isl1/2$^+$ motor neurons at forelimb levels in *msd:Cre*$^+$ *Raldh2*$^{lox/-}$ embryos compared to *Raldh2*$^{lox/-}$ littermates at E12.5, after the peak of motor neuron generation (Nornes and Carry, 1978). We detected a small but statistically significant reduction of approximately 10% in total motor neuron numbers in *msd:Cre*$^+$ *Raldh2*$^{lox/-}$ embryos compared with controls (Fig. S3). Furthermore, the specification of LMC neuronal identity in *msd:Cre*$^+$ *Raldh2*$^{lox/-}$ embryos appeared normal as RALDH2 expression in LMC neurons was not altered and these neurons had not acquired thoracic motor column fates (data not shown).

If reduced RA levels in the spinal cord affect the generation of all motor neurons, the 10% loss of Isl1/2$^+$ neurons should be evenly distributed among all forelimb spinal motor columns of *msd:Cre*$^+$ *Raldh2*$^{lox/-}$ embryos. The number of Isl1/2$^+$/Lhx3$^+$ MMCm neurons was the same between *msd:Cre*$^+$ *Raldh2*$^{lox/-}$ and control embryos (Figs. 2A–C). Similarly, no reduction of Isl/2$^+$/Lhx3$^-$/Lim1$^-$ LMCm neurons was observed (Fig. S3). However, the number of Isl2$^+$/Lim1$^+$ LMCl neurons was reduced by approximately 20% in *msd:Cre*$^+$ *Raldh2*$^{lox/-}$ embryos compared with controls (Figs. 2D–F). Consistent with this, we observed a 40% reduction of an LMCl-specific motor pool marked by expression of the ETS transcription factor Pea3 (Lin et al., 1998; Livet et al., 2002), but no changes were apparent within the larger LMCm Pea3$^+$ motor pool present at forelimb levels (Figs. 2G–I; Fig. S3). In addition, we detected a decrease in the number of Hoxc8 expressing LMCl neurons but Hoxc8 expression in LMCm neurons was unaffected (data not shown). The specific decrease of LMCl neurons was also observed at E11.5, during the peak period of LMCl generation (data not shown). No evidence of increased cell death was detectable by terminal deoxynucleotidyl transferase biotin-dUTP nick end labeling (TUNEL) between *msd:Cre*$^+$ *Raldh2*$^{lox/-}$ and *Raldh2*$^{lox/-}$ controls, consistent with a defect in LMCl generation (data not shown).

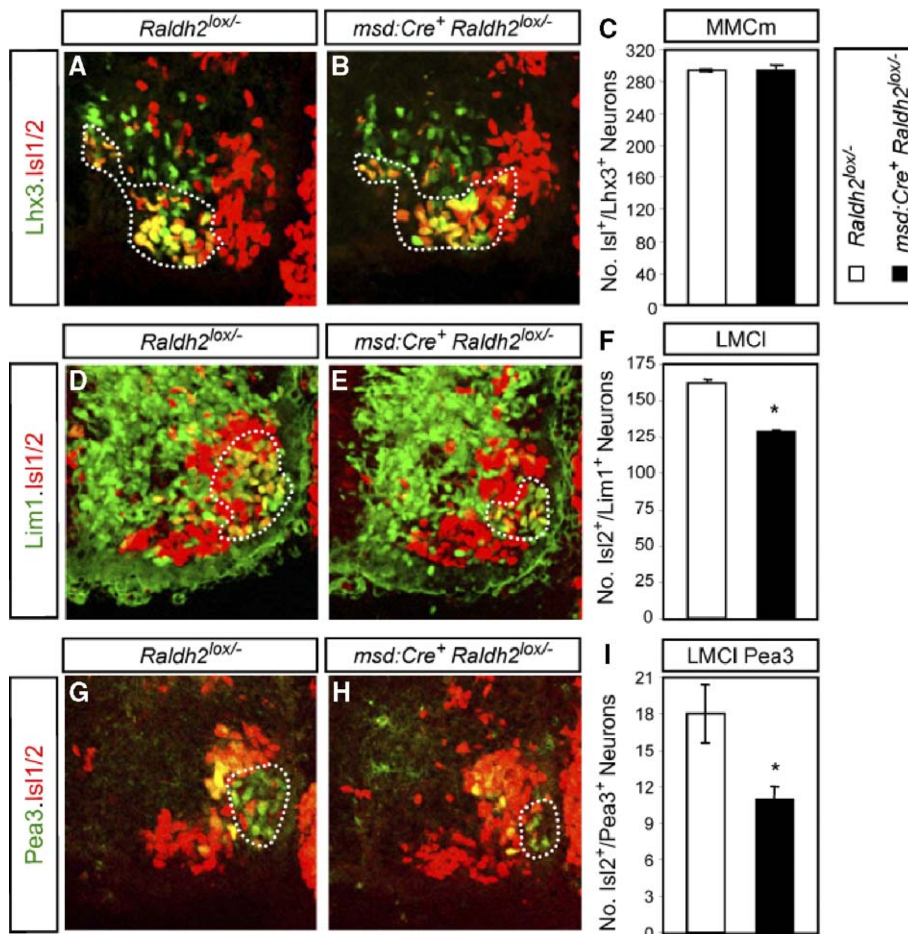

Fig. 2. Forelimb LMCl induction is dependent on paraxial mesoderm-derived RA. Images are confocal micrographs of ventral right quadrants of sectioned mouse forelimb spinal cords at E12.5. In all graphs, mean ± SEM. (A, B) Confocal micrographs of MMCm neurons (yellow cells) outlined by hatched white line. (C) Graph of MMCm neuron numbers (Student's *t* test, $P = 0.471$, $n = 3$ mutants, 4 controls). (D, E) Confocal micrographs of LMCl neurons (yellow cells) outlined by hatched white line. (F) Graph of LMCl neuron numbers (Student's *t* test, $^*P = 0.001$, $n = 3$ mutants, 4 controls) in *msd:Cre*$^+$ *Raldh2*$^{lox/-}$ versus *Raldh2*$^{lox/-}$ siblings. (G, H) Confocal micrographs showing Pea3$^+$ LMCl motor pool outlined by hatched white line. (I) Graph of Pea3$^+$ LMCl neuron numbers (Student's *t* test, $^*P = 0.04$, $n = 3$ mutants, 4 controls).

We next examined if *msd:Cre*$^+$ *Raldh2*$^{lox/-}$ embryos at E12.5 showed similar effects on motor neuron specification at hindlimb levels of the spinal cord. As observed at forelimb regions, the number of Isl1/2$^+$/Lhx3$^+$ MMCm motor neurons was unchanged between *msd:Cre*$^+$ *Raldh2*$^{lox/-}$ embryos and control littermates (Figs. 3A–C). No apparent difference was evident in the number of Isl1/2$^+$/Lhx3$^-$/Lim1$^-$ LMCm neurons (data not shown). However, there was a 14% decrease of Isl2$^+$/Lim1$^+$ LMCl neurons in *msd:Cre*$^+$ *Raldh2*$^{lox/-}$ embryos compared with *Raldh2*$^{lox/-}$ littermates (Figs. 3D–F).

Together, these results indicate that spinal cords of *msd:Cre*$^+$ *Raldh2*$^{lox/-}$ embryos contain enough retinoids to enable motor neurons to form and acquire LMC identity. However, RA levels in these embryos are sufficiently reduced to uncover a requirement for paraxial mesoderm-derived retinoids in specifying LMCl identity at forelimb and hindlimb levels of the spinal cord.

Dorsal axonal projections are compromised in msd:Cre$^+$ *Raldh2*$^{lox/-}$ *embryos*

Besides being molecularly distinct, LMCm and LMCl neurons can be distinguished by their stereotypic projections to ventral and dorsal target muscles respectively in the limb (Hollyday, 1980a,b, 1990; Tosney and Landmesser, 1985a,b). Given that the ablation of RALDH2 in motor neurons elicits a similar 20% decrease of LMCl neurons but also results in the atrophy of distal nerve projections of LMCl neurons (Vermot et al., 2005), we examined if *msd:Cre Raldh2*$^{lox/-}$ mutants showed similar peripheral defects in neurons innervating target muscles in the forelimb at E12.5. In *msd:Cre*$^+$ *Raldh2*$^{lox/-}$ embryos, the initial outgrowth and formation of the dorsal–ventral branches of forelimb-innervating neurons detected by neurofilament staining appeared grossly normal (Fig. 4). However, closer examination showed stunting of the fine branches of dorsally projecting neurons of the radial and ulnar nerves (Figs. 4B–D, F–H), whereas ventrally projecting medial and ulnar nerves remained unaffected (Figs. 4A, E). The penetrance of the phenotype was 100% and was not detected in control embryos. The observation that dorsal and not ventral fine branching is affected in *msd:Cre*$^+$ *Raldh2*$^{lox/-}$ embryos is consistent with perturbations in LMCl and not LMCm development. Moreover, these phenotypes are unlikely to be a result of a general disruption of limb development due to a reduction of RALDH2 in the target, as initial neuronal outgrowth and dorsal–ventral pathways are preserved, ventrally projecting neurons appear normal, and *msd:Cre*$^+$ *Raldh2*$^{lox/-}$ adults do not exhibit digit malformations or abnormal flexure of the distal limbs (data not shown). These results, together with the molecular analyses described above, indicate that mesodermally derived retinoids in the spinal cord are required for the specification of LMCl neurons that innervate dorsal muscles in the limb.

Targeted ablation of RALDH2 in spinal motor neurons

Previous studies suggest that, in addition to LMCl specification, motor neuron sources of RA are required for maintaining the properties of LMCl neurons (Sockanathan and Jessell, 1998). However, this has not been tested in vivo, and it is not known if the maintenance of LMCm neurons is retinoid-dependent. In order to test if LMC-derived retinoids contribute to the maintenance of LMCm and LMCl neurons, we crossed *Raldh2*$^{lox/-}$ mice with

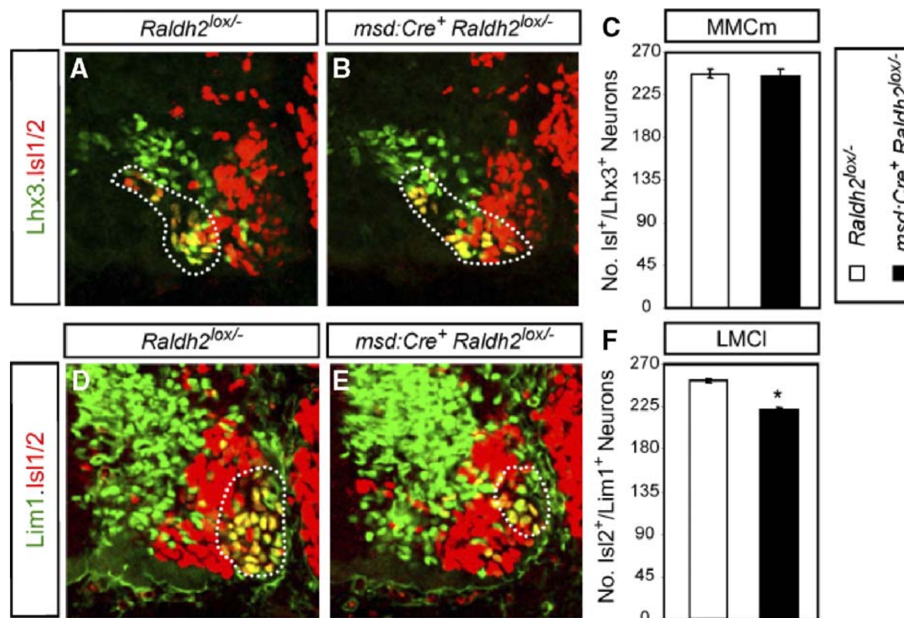

Fig. 3. Hindlimb LMCl induction is dependent on paraxial mesoderm-derived RA. Images are confocal micrographs of ventral right quadrants of sectioned mouse hindlimb spinal cords at E12.5. In all graphs, mean ± SEM. (A, B) Confocal micrographs of MMCm neurons (yellow cells) outlined by hatched white line. (C) Graph of MMCm neuron numbers (Student's *t* test, $P = 0.417$, $n = 3$ mutants, 4 controls). (D, E) Confocal micrographs of LMCl neurons (yellow cells) outlined by hatched white line. (F) Graph of LMCl neuron numbers (Student's *t* test, $^*P = 0.001$, $n = 3$ mutants, 4 controls) in *msd:Cre*$^+$ *Raldh2*$^{lox/-}$ versus *Raldh2*$^{lox/-}$ siblings.

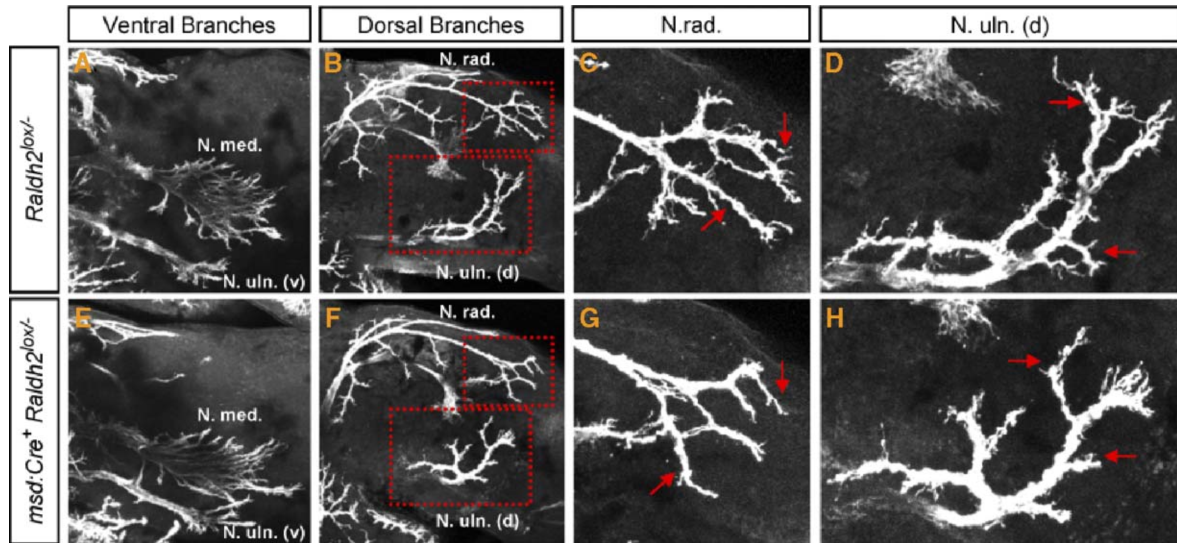

Fig. 4. Dorsal axons innervating the forelimb are abnormal in *msd:Cre*$^{+}$ *Raldh2*$^{lox/-}$ embryos at E12.5. (A–H) Whole mount analyses using neurofilament staining to visualize the main forelimb-innervating neurons in *msd:Cre*$^{+}$ *Raldh2*$^{lox/-}$ and *Raldh2*$^{lox/-}$ littermates by z-stack confocal imaging. Boxes in panel B are shown in higher magnification in panels C and D, boxes in panel F are magnified in panels G and H. Arrows mark the main differences observed. N.rad, radial nerve; N.uln, ulnar nerve; N.med, medial nerve; (d), dorsal; (v) ventral (one representative example of 6 embryos is shown here.)

Isl1:Cre animals. *Isl1:Cre* mice express Cre recombinase within motor neurons and a small population of dorsal interneurons in the spinal cord, but lack expression in the adjacent paraxial mesoderm (Pfaff et al., 1996; Srinivas et al., 2001). Given the temporal and spatial expression of Cre recombinase in this line, effective recombination of the *Raldh2*^{lox} allele in motor neurons is likely to occur after LMCm and LMCl specification has taken place, whereas RALDH2 expression in the paraxial mesoderm should be unaffected. *Isl1:Cre*$^{+}$ *Raldh2*$^{lox/-}$ embryos appear normal, survive to birth and into adulthood and are largely indistinguishable from *Raldh2*$^{lox/-}$ controls.

To assess the extent of RALDH2 loss mediated by Cre recombination in spinal motor neurons, we examined RALDH2 protein by indirect immunofluorescence using RALDH2 specific antibodies in *Isl1:Cre*$^{+}$ *Raldh2*$^{lox/-}$ and *Raldh2*$^{lox/-}$ littermates at E12.5. No difference in mesodermal RALDH2 staining was detected between the embryos; however, a marked loss of RALDH2 expression was consistently observed in LMC neurons of *Isl1:Cre*$^{+}$ *Raldh2*$^{lox/-}$ embryos (Figs. 5A, B). We quantified the amount of RALDH2 protein in forelimb level spinal cords dissected from *Isl1:Cre*$^{+}$ *Raldh2*$^{lox/-}$ and *Raldh2*$^{lox/-}$ littermates by densitometric analysis of Western blots, and found a 60% reduction of RALDH2 protein in Cre^{+} embryos compared to controls (Fig. 5C).

We next determined the extent of RA loss in forelimb-level spinal cords dissected from *Isl1:Cre*$^{+}$ *Raldh2*$^{lox/-}$ and *Raldh2*$^{lox/-}$ littermates at E13.5 using the F9 RARE-LacZ retinoid-responsive reporter cell line (Wagner et al., 1992; McCaffery and Drager, 1994). Ventral spinal cord extracts derived from *Isl1:Cre*$^{+}$ *Raldh2*$^{lox/-}$ embryos were found to have approximately 65% less RA than *Raldh2*$^{lox/-}$ littermates (Fig. 5D). Taken together, these results show that RALDH2 expression and RA levels in postmitotic motor neurons are significantly decreased in *Isl1:Cre*$^{+}$ *Raldh2*$^{lox/-}$ embryos compared with control siblings.

LMCm and LMCl neuronal numbers are dependent upon LMC-derived retinoids

LMCm neuronal specification occurs prior to RALDH2 expression in motor neurons while LMCl induction is partly

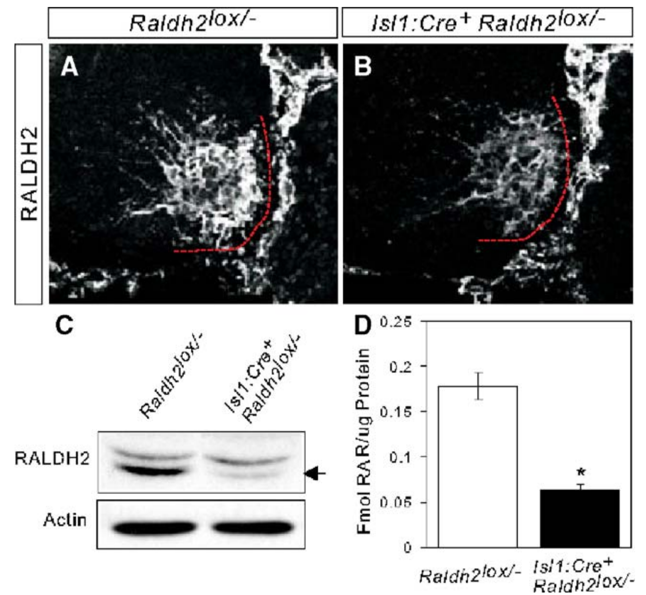

Fig. 5. RALDH2 and RA are reduced in *Isl1:Cre*$^{+}$ *Raldh2*$^{lox/-}$ embryos. (A, B) Confocal micrographs of ventral right quadrants of mouse forelimb spinal cords at E12.5 showing RALDH2 expression. Dotted red line outlines the margins of the spinal cord. (C) Western blot showing reduction of RALDH2 protein in spinal cords of *Isl1:Cre*$^{+}$ *Raldh2*$^{lox/-}$ embryos compared with *Raldh2*$^{lox/-}$ littermates. Actin staining is used to control for amounts of protein loaded per lane. The upper band is a non-specific cross-contaminating band observed when a different extraction buffer was used. (D) Graph depicting the amount of RA released in ventral spinal cord extracts of *Isl1:Cre*$^{+}$ *Raldh2*$^{lox/-}$ E13.5 embryos compared with *Raldh2*$^{lox/-}$ embryos (mean ± SEM, Student's *t* test (D) $^{*}P = 0.006$, $n = 4$ mutants, 3 controls).

dependent upon motor neuron sources of RA. Thus, any decrease of LMCm neurons in *Isl1:Cre*$^{+}$ *Raldh2*$^{lox/-}$ embryos can be attributed to a requirement for RA in LMCm maintenance whereas a decrease in LMCl neurons could potentially arise from an earlier effect on specification. We therefore examined if LMCl specification was altered in *Isl1:Cre*$^{+}$ *Raldh2*$^{lox/-}$ embryos at E11.5 and E12.5, which are developmental time-points spanning the peak period of LMCl specification. No differences in the number of LMCl neurons were found at either time-point, suggesting that LMCl specification is not affected in *Isl1:Cre*$^{+}$ *Raldh2*$^{lox/-}$ embryos compared with control littermates (Fig. S4). The preservation of LMCl specification in these embryos is likely due to sufficient concentrations of RA generated by the paraxial mesoderm, and residual RALDH2 activity in spinal motor neurons. Thus *Isl1:Cre*$^{+}$ *Raldh2*$^{lox/-}$ embryos provide a good in vivo system to investigate the requirement for RA signaling in postmitotic motor neuron maintenance.

In order to assess if motor neuron sources of RA are required to maintain LMCm and LMCl neuronal numbers, we examined *Isl1:Cre*$^{+}$ *Raldh2*$^{lox/-}$ embryos and *Raldh2*$^{lox/-}$ littermates at E13.5, when LMC subtype specification is complete. Analysis of LMCm neurons at forelimb levels showed that the number of Isl^{+}/Lhx3^{-}/Lim1^{-} neurons was reduced by approximately 30% in *Isl1:Cre*$^{+}$ *Raldh2*$^{lox/-}$ embryos compared with *Raldh2*$^{lox/-}$ controls (Figs. 6A–C). Although there was no change in the number of LMCl neurons at E12.5, *Isl1:Cre*$^{+}$ *Raldh2*$^{lox/-}$ embryos showed a 25% reduction of forelimb Isl2^{+}/Lim1^{+} neurons compared with *Raldh2*$^{lox/-}$ controls at E13.5 (Figs. 6A, B, D). A corresponding decrease of the forelimb motor pool markers, Pea3 and Sema3E, that span both LMCm and LMCl columns was also detected (data not shown; Livet et al., 2002). At hindlimb levels, LMCm neuronal numbers showed a decrease of 17% between *Isl1:Cre*$^{+}$ *Raldh2*$^{lox/-}$ and *Raldh2*$^{lox/-}$ embryos (Figs. 6E–G) while LMCl neuronal numbers were reduced by approximately 20% (Figs. 6E, F, H). The smaller decreases in LMCm and LMCl numbers observed at hindlimb levels may be due to temporal effects resulting from the rostral–caudal gradient of maturation of the spinal cord. No changes were observed in the number of forelimb or hindlimb MMCm neurons (data not shown). Taken together, these results indicate that retinoids synthesized by spinal motor neurons are required for the maintenance of LMCm and LMCl neuronal numbers.

Isl1:Cre^{+} *Raldh2*$^{lox/-}$ embryos show abnormal axonal projections and loss of Hoxc8

Given the decrease of LMCm and LMCl neurons in *Isl1:Cre*$^{+}$ *Raldh2*$^{lox/-}$embryos, we examined if the patterns of axonal projections of limb-innervating neurons in these embryos were affected by reduced levels of RA in motor neurons. In *Isl1:Cre*$^{+}$ *Raldh2*$^{lox/-}$ embryos at E13.5, the overall outgrowth and formation of the dorsal and ventral axonal branches within the forelimb appeared similar to *Raldh2*$^{lox/-}$ controls. However, we found that both ventral and dorsal projecting neurons were stunted or atrophied and in some cases appeared retracted (Figs. 7A–F). These peripheral defects are more severe than the abnormalities observed in *msd:Cre*$^{+}$ *Raldh2*$^{lox/-}$ embryos and may reflect an erosion of the properties of these remaining LMC neurons.

Mice lacking the homeotic protein Hoxc8, show incomplete LMCl specification, but also show increased apoptosis of LMC neurons and altered topographic maps of motor pools (Tiret et al., 1998; Vermot et al., 2005). It is therefore possible that the defects observed in *Isl1:Cre*$^{+}$ *Raldh2*$^{lox/-}$ embryos result from the inability to maintain Hoxc8 expression in motor neurons due to

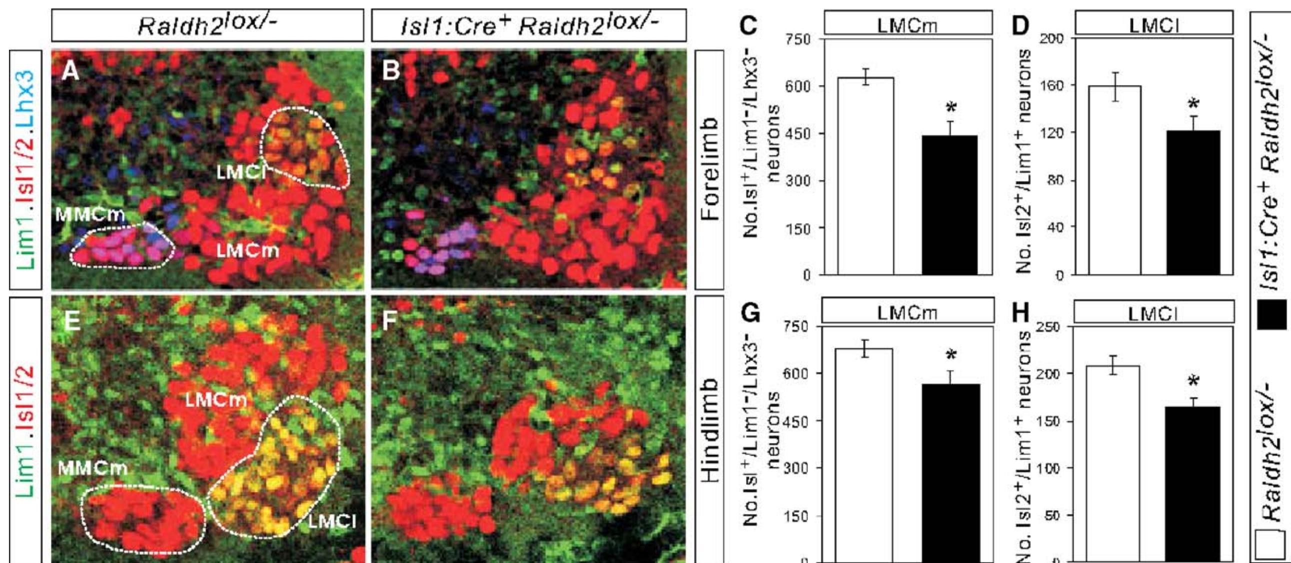

Fig. 6. LMCm and LMCl neurons are reduced in *Isl1:Cre*$^{+}$ *Raldh2*$^{lox/-}$ embryos at E13.5. (A, B) Confocal micrographs of ventral right quadrants of sectioned mouse forelimb E13.5 spinal cord. LMCl (orange), LMCm (red) and MMCm (purple) are marked. (C, D) Graphs quantifying LMCm (mean ± SEM, Student's *t* test, **P* = 0.03, *n* = 4 mutants, 3 controls) and LMCl neurons (mean ± SEM, Student's *t* test, **P* = 0.03, *n* = 4 mutants, 3 controls) in *Raldh2*$^{lox/-}$ and *Isl1:Cre*$^{+}$ *Raldh2*$^{lox/-}$ littermates. (E, F) Confocal micrographs of ventral right quadrants of sectioned mouse hindlimb E13.5 spinal cord. (G, H) Graphs quantifying LMCm (mean ± SEM, Student's *t* test, **P* = 0.03, *n* = 4 mutants, 6 controls) and LMCl neurons (mean ± SEM, Student's *t* test, **P* = 0.008, *n* = 4 mutants, 6 controls) in *Raldh2*$^{lox/-}$ and *Isl1:Cre*$^{+}$ *Raldh2*$^{lox/-}$ controls. Hatched white lines outline MMCm and LMCl motor neurons.

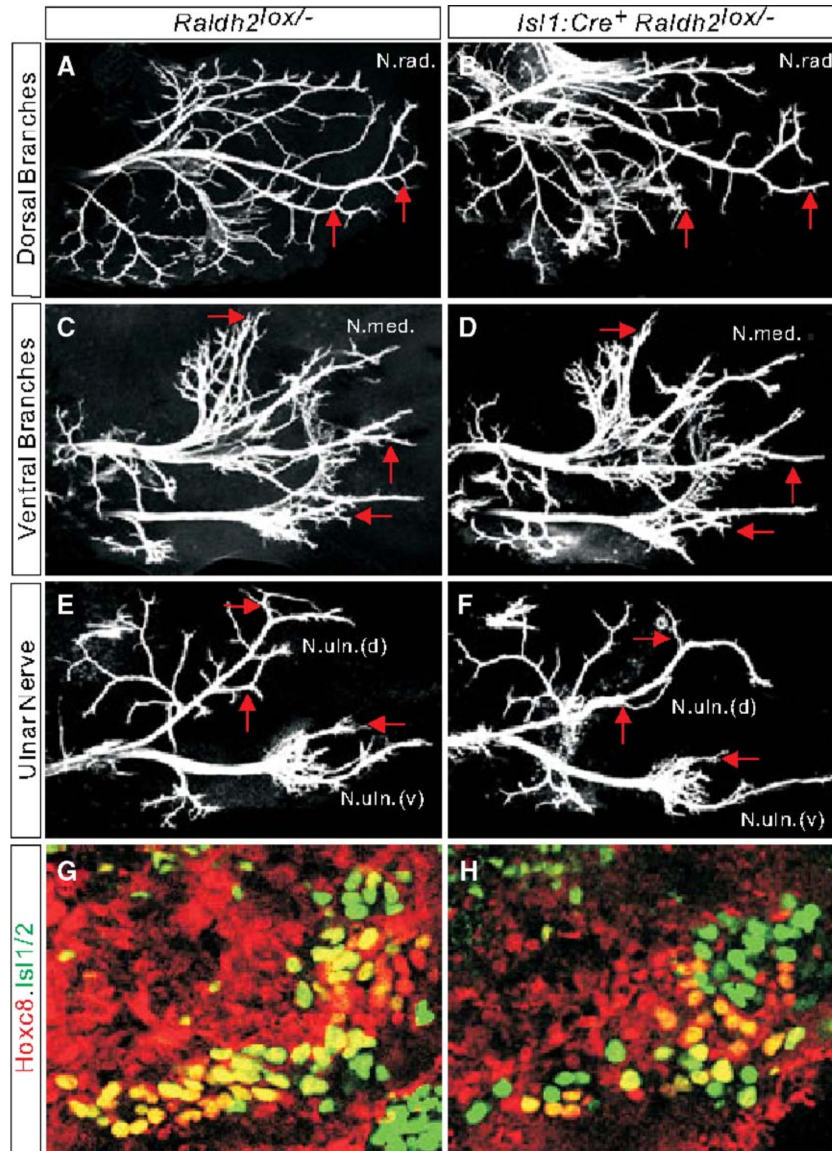

Fig. 7. Axonal projections and Hoxc8 expression at E13.5 in *Isl1:Cre*$^+$ *Raldh2*$^{lox/-}$ embryos. (A–F) Whole mount analyses using neurofilament staining to visualize the main forelimb-innervating axons in *Raldh2*$^{lox/-}$ and *Isl1:Cre*$^+$ *Raldh2*$^{lox/-}$ littermates by z-stack confocal imaging. Arrows mark the main differences observed. N.rad, radial nerve; N.uln, ulnar nerve; N.med, medial nerve; (d), dorsal; (v) ventral. Data are representative of 3 independent experiments. (G, H) Confocal micrographs of ventral right quadrants of mouse forelimb spinal cords showing decrease of Hoxc8 expression in motor neurons of *Isl1:Cre*$^+$ *Raldh2*$^{lox/-}$ embryos.

decreased levels of RA synthesized in these cells. Analysis of Hoxc8 expression in forelimb spinal motor neurons of E13.5 *Isl1:Cre*$^+$ *Raldh2*$^{lox/-}$ embryos showed fewer Hoxc8$^+$ motor neurons compared with controls, and this loss corresponded to the decrease of LMCm and LMCl neurons described earlier. In addition, the levels of Hoxc8 expression were significantly decreased in the majority of LMCm and LMCl neurons that remained (Figs. 7G, H). These observations suggest that sustained expression of Hoxc8 in motor neurons by RA-dependent mechanisms may be required for the maintenance of limb-innervating spinal motor neurons.

Discussion

LMCm and LMCl motor neurons are generated specifically at limb levels of the spinal cord and project their axons to ventral and dorsal limb muscles, respectively (Jessell, 2000; Shirasaki and Pfaff, 2002; Price and Briscoe, 2004). Previous studies focusing on forelimb LMC specification have implicated retinoids synthesized by early born LMCm neurons as being necessary for inducing the identity of late born LMCl neurons (Sockanathan and Jessell, 1998; Vermot et al., 2005, Fig. 8A). Using mouse genetics, we show here that RA synthesized in the paraxial mesoderm functions as a second source of signals to specify LMCl neurons at both forelimb and hindlimb levels of the spinal cord (Fig. 8B). Furthermore, we define later roles for retinoids synthesized by motor neurons in maintaining appropriate numbers of LMCm and LMCl cells, and in preserving the axonal integrity of these neurons (Fig. 8C). These studies suggest that mesodermal and neuronal retinoid sources act coordinately to establish and maintain appropriate cohorts of limb-innervating motor neurons in the spinal cord.

Paraxial mesoderm-derived retinoids and LMC subtype specification

Studies in the chick have led to a model of LMCl specification where early born LMCm neurons express RALDH2 and provide a motor neuron source of RA at limb levels of the spinal cord. Late born LMC cells are exposed to these RA signals as they migrate through the LMCm population to reach their lateral settling position, and consequently acquire LMCl neuronal identities (Sockanathan and Jessell, 1998). In support of this model, a recent study inactivating RALDH2 in motor neurons at forelimb levels resulted in a 20% loss of LMCl neurons (Vermot et al., 2005). However, a significant number of LMCl neurons are still generated in these animals, indicating that other signals are required to specify the normal complement of LMCl neurons. Moreover, the rostral–caudal restriction of Cre expression in these animals prevents the analysis of LMCl specification at hindlimb levels of the spinal cord, restricting the authors' analysis to forelimb regions.

Retinoids synthesized by RALDH2 in the paraxial mesoderm are known to contribute significantly to RA levels within the spinal cord (Mic et al., 2002; Niederreither et al., 2002). Consequently, mesodermally derived retinoids may potentially provide an additional source of signals to specify LMCl neuronal identity. One approach to test this hypothesis is to ablate RALDH2 expression in the paraxial mesoderm and examine if LMCl specification is compromised. However, studies in the chick and in Vitamin A-deficient animals have identified a requirement for mesodermally derived retinoids in the generation of generic motor neurons, and the imposition of forelimb LMC columnar identity (Novitch et al., 2003; Diez del Corral et al., 2003; Sockanathan et al., 2003). Thus, complete ablation of RA in the mesoderm is likely to result in either a severe loss of motor neurons or the adoption of thoracic motor column fates, confounding an analysis of LMCl specification. In our study, *msd:Cre*$^{+}$ *Raldh2*$^{lox/-}$ mice exhibit a 50% reduction of RALDH2 protein in the paraxial mesoderm and a concomitant 50% loss of RA levels within the spinal cord. This degree of RA loss does not affect either generic motor neuron generation or the acquisition of forelimb LMC character. Further, *msd:Cre*$^{+}$ *Raldh2*$^{lox/-}$ embryos have equivalent numbers of LMCm neurons compared with controls and show no changes in LMC RALDH2 expression, suggesting that motor neuron sources of RA are intact in these animals. In addition, other sources of RA synthetic activity within the spinal cord are unchanged between *msd:Cre*$^{+}$ *Raldh2*$^{lox/-}$ embryos and controls. Taken together, *msd:Cre*$^{+}$ *Raldh2*$^{lox/-}$ mice provide a useful model to test if retinoids from the adjacent paraxial mesoderm are required for the specification of LMCl neurons in vivo. An added advantage of these animals is that both forelimb and hindlimb LMCl development can be assessed.

We find that, in *msd:Cre*$^{+}$ *Raldh2*$^{lox/-}$ embryos, the number of LMCl neurons is decreased at both forelimb and hindlimb levels of the spinal cord, whereas LMCm and MMCm neurons are unaffected. Thus, our study indicates that retinoids derived from the paraxial mesoderm provide a second source of RA to establish the normal complement of LMCl neurons, and these signals operate at both forelimb and hindlimb levels. We suggest a model where LMCl specification is dependent upon paracrine retinoid signals, and the contribution of mesodermal and neuronal sources differs depending on their relative contributions to RA levels within the spinal cord at a given time (Fig. 8B). Thus, at early developmental stages, prior to the establishment of a critical number of RALDH2 expressing LMCm neurons, paraxial mesoderm-derived retinoids may provide the main source of retinoids that specify LMCl neurons in vivo. When mesodermal RA sources decline and LMCm neurons increase in number, RA synthesized by motor neurons plays a more prominent role in specifying LMCl identities. This interplay provides another example where mesodermal signals initiate cell fate specification events in the spinal cord prior to the consolidation of a second signaling center that operates within the neural tube itself (Jessell, 2000).

Motor neuron-derived retinoids and the maintenance of LMCm and LMCl neurons

Chick neural explant studies have suggested that RA signaling is required for the maintenance of LMCl neurons (Sockanathan and Jessell, 1998); but, a potential role for retinoids in motor neuron maintenance has not been tested

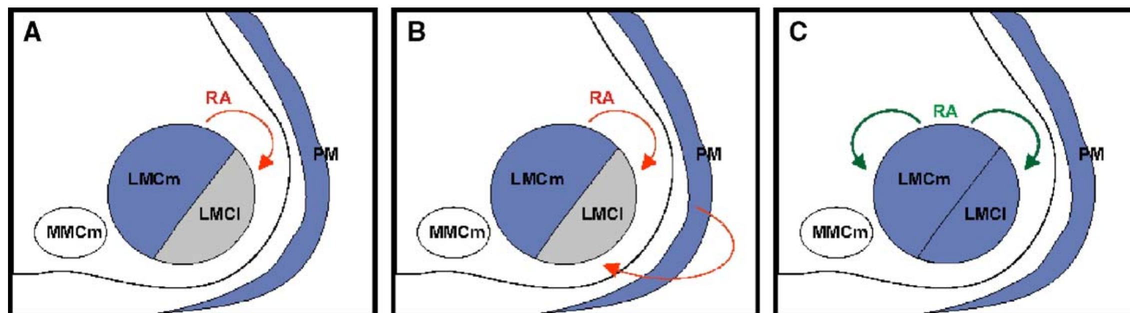

Fig. 8. Model for RA signaling in LMCl specification and LMC maintenance. RA signals from early born LMCm neurons expressing RALDH2 (blue) are required for LMCl specification (red arrows, A, B). However, RA derived from RALDH2 expression in the adjacent paraxial mesoderm (PM, blue) also functions to induce LMCl identity and may form the primary source of RA prior to the establishment of sufficient local concentrations of retinoids by LMCm neurons (red arrow, B). After specification is complete, both LMCm and LMCl neurons express RALDH2 (blue) and synthesize RA, which is required to maintain LMCm and LMCl neuronal numbers but not that of adjacent MMCm neurons (green arrows, C).

genetically in mammalian model systems. By reducing RALDH2 levels in motor neurons once they are postmitotic, we find that RA synthesized by motor neurons is required for maintaining forelimb and hindlimb LMCm and LMCl neuronal numbers, whereas adjacent MMCm neurons are unaffected. Interestingly, no effect on LMCm numbers is observed in *msd:Cre*$^{+}$ *Raldh2*$^{lox/-}$ embryos although the spinal cords of these embryos have lower overall RA levels compared with *Isl1:Cre*$^{+}$ *Raldh2*$^{lox/-}$ embryos (50% versus a 35% reduction, S.J. and S.S. unpublished data). This observation suggests that local, short-range RA signals may operate to maintain LMC numbers and raises the possibility that this might occur through cell-autonomous mechanisms.

Our studies suggest that genes downstream of RA signals act specifically to maintain spinal motor neurons that innervate the limb. What are the molecules that mediate motor neuron maintenance? In the case of LMC neurons, we suggest that Hoxc8 may be required to maintain LMC neurons at posterior forelimb levels of the spinal cord. Interestingly, Hoxc8 also acts earlier to specify LMCl identity (Vermot et al., 2005). This dual role for Hoxc8 is supported by analyses of Hoxc8 knockout mice which show defects in motor neuron specification, altered somatotopic projection maps and increased cell death (Tiret et al., 1998; Vermot et al., 2005). Hoxc8 expression in motor neurons is restricted to the C7, C8 and T1 regions at forelimb levels of the spinal cord (Tiret et al., 1998; Dasen et al., 2003) suggesting that other proteins must mediate the maintenance of anterior forelimb and hindlimb LMC neurons. Since the expression of Hox gene clusters span distinct regions of the spinal cord, it is possible that different Hox proteins may function downstream of RA signaling to maintain LMC neurons at other axial levels (Carpenter, 2002). Of note, Hox proteins involved in fate specification may not always have a second function in motor neuron maintenance. Two examples are Hoxd10 and Hoxa10 which are expressed in lumbar LMC neurons, have roles in hindlimb LMC specification (Lin and Carpenter, 2003; Shah et al., 2004) but are largely down-regulated in spinal motor neurons at later stages of development (Choe et al., 2006).

What are the cellular processes affecting maintenance that might be impacted by decreased retinoid signaling in motor neurons? Potential pathways include those mediating programmed cell-death and cell-survival through the action of target-derived neurotrophic factors (Buss and Oppenheim, 2004; Zweifel et al., 2005). Neurotrophic factors such as NT3 and NGF bind to members of the Trk family of receptors to mediate cell survival, partly through the antagonism of cell death pathways (Zweifel et al., 2005). RA has the capability to regulate and stabilize the expression of TrkA, TrkB and p75 receptors in cell lines raising the compelling possibility that RA signaling may mediate motor neuron survival through similar mechanisms in vivo (Scheibe and Wagner, 1992; Lucarelli et al., 1995; Xie et al., 1997). Interestingly, the requirement for RA signaling in motor neurons may extend to adulthood, as adult rats deprived of Vitamin A undergo motor neuron degeneration (Corcoran et al., 2002). Although in this case, the loss of RA is not confined to motor neurons, it is possible that continuous RA synthesis in motor neurons is necessary for the maintenance and survival of motor neurons throughout life.

In summary, we have utilized genetic approaches to define new roles for mesodermal and motor neuron sources of RA in the specification and maintenance of limb-innervating motor neurons of the spinal cord. This work leads to a revised model of LMCm and LMCl development, where RAs in the spinal cord derived from adjacent paraxial mesoderm and motor neurons function together to specify the correct complement of LMCl neurons. Retinoids synthesized by motor neurons have additional roles later in development to maintain appropriate numbers of LMCm and LMCl neurons. The identification of retinoid-responsive downstream pathways that mediate these critical events in motor neuron specification and maintenance will be essential to understand the molecular mechanisms by which retinoids govern neuronal identity, maintenance and survival.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ydbio.2006.05.015.

References

Arber, S., Han, B., Mendelsohn, M., Smith, M., Jessell, T.M., Sockanathan, S., 1999. Requirement for the homeobox gene Hb9 in the consolidation of motor neuron identity. Neuron 23, 659–674.

Beckers, J., Caron, A., Hrabe de Angelis, M., Hans, S., Campos-Ortega, J.A., Gossler, A., 2000. Distinct regulatory elements direct delta1 expression in the nervous system and paraxial mesoderm of transgenic mice. Mech. Dev. 95, 23–34.

Berggren, K., McCaffery, P., Drager, U., Forehand, C.J., 1999. Differential distribution of retinoic acid synthesis in the chicken embryo as determined by immunolocalization of the retinoic acid synthetic enzyme, RALDH2. Dev. Biol. 210, 288–304.

Blentic, A., Gale, E., Maden, M., 2003. Retinoic acid signalling centres in the avian embryo identified by sites of expression of synthesising and catabolising enzymes. Dev. Dyn. 227, 114–127.

Buss, R.R., Oppenheim, R.W., 2004. Role of programmed cell death in normal neuronal development and function. Anat. Sci. Int. 79, 191–197.

Carpenter, E.M., 2002. Hox genes and spinal cord development. Dev. Neurosci. 24, 24–34.

Choe, A., Phun, H.Q., Tieu, D.D., Hu, Y.H., Carpenter, E.M., 2006. Expression patterns of Hox10 paralogous genes during lumbar spinal cord development. Gene Expr. Patterns, doi:10.1016/j.modgep.2005.12.004.

Corcoran, J., So, P.L., Maden, M., 2002. Absence of retinoids can induce

motoneuron disease in the adult rat and a retinoid defect is present in motoneuron disease patients. J. Cell Sci. 115, 4735–4741.
Cornbrooks, E.B., Newton, C.J., Forehand, C.J., 1997. Development of differential preganglionic projections to pre- and paravertebral sympathetic ganglia. J. Comp. Neurol. 382, 1–18.
Dasen, J.S., Liu, J.P., Jessell, T.M., 2003. Motor neuron columnar fate imposed by sequential phases of Hox-c activity. Nature 425, 926–933.
Diez del Corral, R., Olivera-Martinez, I., Goriely, A., Gale, E., Maden, M., Storey, K., 2003. Opposing FGF and retinoid pathways control ventral neural pattern, neuronal differentiation, and segmentation during body axis extension. Neuron 40, 65–79.
Giger, R.J., Cloutier, J.F., Sahay, A., Prinjha, R.K., Levengood, D.V., Moore, S.E., Pickering, S., Simmons, D., Rastan, S., Walsh, F.S., Kolodkin, A.L., Ginty, D.D., Geppert, M., 2000. Neuropilin-2 is required in vivo for selective axon guidance responses to secreted semaphorins. Neuron 25, 29–41.
Gutman, C.R., Ajmera, M.K., Hollyday, M., 1993. Organization of motor pools supplying axial muscles in the chicken. Brain Res. 609, 129–136.
Haselbeck, R.J., Ang, H.L., Deltour, L., Duester, G., 1997. Retinoic acid and alcohol/retinol dehydrogenase in the mouse adrenal gland: a potential endocrine source of retinoic acid during development. Endocrinology 138, 3035–3041.
Hollyday, M., 1980a. Organization of motor pools in the chick lumbar lateral motor column. J. Comp. Neurol. 194, 143–170.
Hollyday, M., 1980b. Motoneuron histogenesis and the development of limb innervation. Curr. Top. Dev. Biol. 15, 181–215.
Hollyday, M., 1990. Specificity of initial axonal projections to embryonic chick wing. J. Comp. Neurol. 302, 589–602.
Jessell, T.M., 2000. Neuronal specification in the spinal cord: inductive signals and transcriptional codes. Nat. Rev., Genet. 1, 20–29.
Jessell, T.M., Melton, D.A., 1992. Diffusible factors in vertebrate embryonic induction. Cell 68, 257–270.
Kania, A., Johnson, R.L., Jessell, T.M., 2000. Coordinate roles for LIM homeobox genes in directing the dorsoventral trajectory of motor axons in the vertebrate limb. Cell 102, 161–173.
Landmesser, L., 1978. The distribution of motor neurons supplying the chick hind limb muscles. J. Physiol. 284, 371–389.
Lin, A.W., Carpenter, E.M., 2003. Hoxa10 and Hoxd10 coordinately regulate lumbar motor neuron patterning. J. Neurobiol. 56, 328–337.
Lin, J.H., Saito, T., Anderson, D.J., Lance-Jones, C., Jessell, T.M., Arber, S., 1998. Functionally related motor neuron pool and muscle sensory afferent subtypes defined by coordinate ETS gene expression. Cell 95, 393–407.
Liu, A., Joyner, A.L., 2001. Early anterior/posterior patterning of the midbrain and cerebellum. Annu. Rev. Neurosci. 24, 869–896.
Livet, J., Sigrist, M., Stroebel, S., De Paola, V., Price, S.R., Henderson, C.E., Jessell, T.M., Arber, S.A., 2002. ETS gene Pea3 controls the central position and terminal arborization of specific motor neuron pools. Neuron 35, 877–892.
Lucarelli, E., Kaplan, D.R., Thiele, C.J., 1995. Selective regulation of TrkA and TrkB receptors by retinoic acid and interferon-gamma in human neuroblastoma cell lines. J. Biol. Chem. 270, 24725–24731.
McCaffery, P., Drager, U.C., 1994. Hot spots of retinoic acid synthesis in the developing spinal cord. Proc. Natl. Acad. Sci. U. S. A. 91, 7194–7197.
Mic, F.A., Haselbeck, R.J., Cuenca, A.E., Duester, G., 2002. Novel retinoic acid generating activities in the neural tube and heart identified by conditional rescue of Raldh2 null mutant mice. Development 129, 2271–2282.
Niederreither, K., McCaffery, P., Drager, U.C., Chambon, P., Dolle, P., 1997. Restricted expression and retinoic acid induced downregulation of the retinaldehyde dehydrogenase type 2 (RALDH-2) gene during mouse development. Mech. Dev. 62, 67–78.
Niederreither, K., Subbarayan, V., Dolle, P., Chambon, P., 1999. Embryonic retinoic acid synthesis is essential for early mouse post-implantation development. Nat. Genet. 21, 444–448.
Niederreither, K., Vermot, J., Fraulob, V., Chambon, P., Dolle, P., 2002. Retinaldehyde dehydrogenase 2 (RALDH2)-independent patterns of retinoic acid synthesis in the mouse embryo. Proc. Natl. Acad. Sci. U. S. A. 99, 16111–16116.
Nornes, H.O., Carry, M., 1978. Neurogenesis in spinal cord of mouse: an autoradiographic analysis. Brain Res. 159, 1–6.
Novitch, B.G., Wichterle, H., Jessell, T.M., Sockanathan, S., 2003. A requirement for retinoic acid-mediated transcriptional activation in ventral neural patterning and motor neuron specification. Neuron 40, 81–95.
Pfaff, S.L., Mendelsohn, M., Stewart, C.L., Edlund, T., Jessell, T.M., 1996. Requirement for LIM homeobox gene Isl1 in motor neuron generation reveals a motor neuron-dependent step in interneuron differentiation. Cell 84, 309–320.
Prasad, A., Hollyday, M., 1991. Development and migration of avian sympathetic preganglionic neurons. J. Comp. Neurol. 307, 237–258.
Price, S.R., Briscoe, J., 2004. The generation and diversification of spinal motor neurons: signals and responses. Mech. Dev. 121, 1103–1115.
Rajewsky, K., Gu, H., Kuhn, R., Betz, U.A., Muller, W., Roes, J., Schwenk, F., 1996. Conditional gene targeting. J. Clin. Invest. 98, 600–603.
Rossant, J., Zirngibl, R., Cado, D., Shago, M., Giguere, V., 1991. Expression of a retinoic acid response element-hsplacZ transgene defines specific domains of transcriptional activity during mouse embryogenesis. Genes Dev. 5, 1333–1344.
Schaeren-Wiemers, N., Gerfin-Moser, A., 1993. A single protocol to detect transcripts of various types and expression levels in neural tissue and cultured cells: in situ hybridization using digoxigenin-labelled cRNA probes. Histochemistry 100, 431–440.
Scheibe, R.J., Wagner, J.A., 1992. Retinoic acid regulates both expression of the nerve growth factor receptor and sensitivity to nerve growth factor. J. Biol. Chem. 267, 17611–17616.
Shah, V., Drill, E., Lance-Jones, C., 2004. Ectopic expression of Hoxd10 in thoracic spinal segments induces motoneurons with a lumbosacral molecular profile and axon projections to the limb. Dev. Dyn. 231, 43–56.
Sharma, K., Sheng, H.Z., Lettieri, K., Li, H., Karavanov, A., Potter, S., Westphal, H., Pfaff, S.L., 1998. LIM homeodomain factors Lhx3 and Lhx4 assign subtype identities for motor neurons. Cell 95, 817–828.
Sharma, K., Leonard, A.E., Lettieri, K., Pfaff, S.L., 2000. Genetic and epigenetic mechanisms contribute to motor neuron pathfinding. Nature 406, 515–519.
Shirasaki, R., Pfaff, S.M., 2002. Transcriptional controls and the control of neuronal identity. Annu. Rev. Neurosci. 25, 251–281.
Sockanathan, S., Jessell, T.M., 1998. Motor neuron-derived retinoid signaling specifies the subtype identity of spinal motor neurons. Cell 94, 503–514.
Sockanathan, S., Perlmann, T., Jessell, T.M., 2003. Retinoid receptor signaling in postmitotic motor neurons regulates rostrocaudal positional identity and axonal projection pattern. Neuron 40, 97–111.
Solomin, L., Johansson, C.B., Zetterstrom, R.H., Bissonette, R.P., Heyman, R.A., Olson, L., Lendahl, U., Frisen, J., Perlmann, T., 1998. Retinoid X receptor signaling in the developing spinal cord. Nature 395, 398–402.
Soriano, P., 1999. Generalized lacZ expression with the ROSA26 Cre reporter strain. Nat. Genet. 21, 70–71.
Srinivas, S., Watanabe, T., Lin, C.S., William, C.M., Tanabe, Y., Jessell, T.M., Costantini, F., 2001. Cre reporter strains produced by targeted insertion of EYFP and ECFP into the ROSA26 locus. BMC Dev. Biol. 1, 4.
Swindell, E.C., Thaller, C., Sockanathan, S., Petkovich, M., Jessell, T.M., Eichele, G., 1999. Complementary domains of retinoic acid production and degradation in the early chick embryo. Dev. Biol. 216, 282–296.
Tiret, L., Le Mouellic, H., Maury, M., Brulet, P., 1998. Increased apoptosis of motoneurons and altered somatotopic maps in the brachial spinal cord of Hoxc-8-deficient mice. Development 125, 279–291.
Tosney, K.W., Landmesser, L.T., 1985a. Development of the major pathways for neurite outgrowth in the chick hindlimb. Dev. Biol. 109, 193–214.
Tosney, K.W., Landmesser, L.T., 1985b. Growth cone morphology and trajectory in the lumbosacral region of the chick embryo. J. Neurosci. 5, 2345–2358.
Tsuchida, T.N., Ensini, M., Morton, S.B., Baldessare, M., Edlund, T., Jessell, T.M., Pfaff, S.L., 1994. Topographic organization of embryonic motor neurons defined by expression of LIM homeobox genes. Cell 79, 957–970.
Vermot, J., Niederreither, K., Garnier, J.M., Chambon, P., Dolle, P., 2003. Decreased embryonic retinoic acid synthesis results in a DiGeorge syndrome phenotype in newborn mice. Proc. Natl. Acad. Sci. U. S. A. 100, 1763–1768.

Vermot, J., Schuhbaur, B., Le Mouellic, H., McCaffery, P., Garnier, J.M., Hentsch, D., Brulet, P., Niederreither, K., Chambon, P., Dolle, P., Le Roux, I., 2005. Retinaldehyde dehydrogenase 2 and Hoxc8 are required in the murine brachial spinal cord for the specification of Lim1+ motoneurons and the correct distribution of Islet1+ motoneurons. Development 132, 1611–1621.

Wagner, M., Han, B., Jessell, T.M., 1992. Regional differences in retinoid release from embryonic neural tissue detected by an in vitro reporter assay. Development 116, 55–66.

Wilson, L., Gale, E., Chambers, D., Maden, M., 2004. Retinoic acid and the control of dorsoventral patterning in the avian spinal cord. Dev. Biol. 269, 433–446.

Xie, P., Cheung, W.M., Ip, F.C., Ip, N.Y., Leung, M.F., 1997. Induction of TrkA receptor by retinoic acid in leukaemia cell lines. NeuroReport 8, 1067–1070.

Zhao, D., McCaffery, P., Ivins, K.J., Neve, R.L., Hogan, P., Chin, W.W., Drager, U.C., 1996. Molecular identification of a major retinoic acid synthesizing enzyme, a retinaldehyde-specific dehydrogenase. Eur. J. Biochem. 240, 15–22.

Zweifel, L.S., Kuruvilla, R., Ginty, D.D., 2005. Functions and mechanisms of retrograde neurotrophin signalling. Nat. Rev., Neurosci. 6, 615–625.