Retinoic Acid-Dependent Signaling Pathways and Lineage Events in the Developing Mouse Spinal Cord

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Abstract

Studies in avian models have demonstrated an involvement of retinoid signaling in early neural tube patterning. The roles of this signaling pathway at later stages of spinal cord development are only partly characterized. Here we use Raldh2-null mouse mutants rescued from early embryonic lethality to study the consequences of lack of endogenous retinoid acid (RA) in the differentiating spinal cord. Mid-gestation RA deficiency produces prominent structural and molecular deficiencies in dorsal regions of the spinal cord. While targets of Wnt signaling in the dorsal neuronal lineage are unaltered, reductions in Fibroblast Growth Factor (FGF) and Notch signaling are clearly observed. We further provide evidence that endogenous RA is capable of driving stem cell differentiation. Raldh2 deficiency results in a decreased number of spinal cord derived neurospheres, which exhibit a reduced differentiation potential. Raldh2-null neurospheres have a decreased number of cells expressing the neuronal marker β III-tubulin, while the nestin-positive cell population is increased. Hence, in vivo retinoid deficiency impaired neural stem cell growth. We propose that RA has separable functions in the developing spinal cord to (i) maintain high levels of FGF and Notch signaling and (ii) drive stem cell differentiation, thus restricting both the numbers and the pluripotent character of neural stem cells.

Introduction

Spinal cord development and neurogenesis are extremely interesting not only in the context of spinal injury and regeneration, but also in terms of the basic developmental processes that control cell lineage and specification. Complex molecular mechanisms guide progenitors cells in a specific spatial order that depend on their location according to the embryonic anteroposterior (AP) and dorsoventral (DV) axes. How patterning signals determine the identity of neural progenitors in a precise spatio-temporal order is still an issue of extensive study. Amongst the molecular pathways involved in this process is the retinoic acid (RA) pathway, which has multiple stage-specific functions in the generation, patterning, and maintenance of neural tissues [1,2]. Initially neuroepithelial progenitors reside in a caudal neural plate ‘stem zone’ in which fibroblast growth factors (FGFs) act permissively to allow neural stem cell expansion [3,4,5], and initial stages of neural specification [6]. At these early stages RA appears to coordinate the progressive maturation of spinal cord progenitors (reviewed in [7]). RA synthesized by retinaldehyde dehydrogenase 2 (RALDH2) in differentiating paraxial (presomitic and somitic) mesoderm, diffuses towards the neuroepithelium and differentiates progenitor motor neurons (pMN) as they emerge from the caudal stem cell zone. Inhibiting RA signaling expands the size of the progenitor zone, seen as an enlarged caudal Fgfb expression zone [8,9]. Retinoid deficiency inhibits the initiation of pMN differentiation, affecting Pax6, Olig2, Ngn2 and Bhlhb5 expression [10,11].

The next steps in dorsoventral pMN patterning require ventral Sonic hedgehog (Shh) and dorsal Wnts secretion (reviewed in [12,13]). The morphogenic action of these main signals differentially induces ventral or dorsal transcriptional targets, leading to the establishment of neuronal subtypes, so neuronal circuits can form and function independent of their initial inducers (reviewed in [14,15]). Retinoic acid is intricately involved in many steps in this process (reviewed in [2]). Data on vitamin A-deficient quail embryos indicate that RA signaling is required for expression of dorsal patterning genes, playing additional roles in pMN and interneuron specification [16]. Retinoid roles at later stages include guiding the induction and patterning of lateral motor column (LMC) neurons [17] by regulating AP patterning genes including Hox genes [18]. Viable, tissue-specific mutants for the RA-synthesizing enzyme RALDH2 exhibit a reduced population of Lim1-positive brachial motoneurons, mispositioned LMC Islet1-positive neurons, and disregulated Hox6, leading to inappropriate axonal projection of nerves innervating extensor muscles and forelimb paralysis defects [17].
We have investigated these potential functions of RA by analyzing the spinal cord of Raldh2\(^{-/-}\) null mutants rescued from early embryonic lethality by transient maternal RA supplementation [19,20]. We show that dorsal spinal cord growth deficits are not due to abnormal Wnt- or dorsal-specific progenitor transcript levels. Rather, RA-deficient spinal cords are characterized by reduced dorsal FGF signaling and impaired expression of several Notch effectors. As a consequence, RA-deficiency inhibits neuronal stem cell proliferation, impairing neurosphere growth, differentiation and radial glial expression. Cell sorting experiments further show an expansion of the `side population` (SP) of putative stem cells in the retinoid-deficient spinal cord. According to their transcriptional profiles, these cells were diverted from differentiation towards radial glia and maintained as pluripotent precursors and/or neural stem cells. In addition, analysis of spinal cord-derivated neurospheres indicates that RA promotes neuronal differentiation versus pluripotent precursor maintenance.

**Results**

**Rescued Raldh2\(^{-/-}\) mutants as a model for RA deficiency in the differentiating spinal cord**

To analyze RA-dependent events in the differentiating mouse spinal cord, we took advantage of a rescue system allowing to postpone the lethality of the Raldh2\(^{-/-}\) mutants (occurring at embryonic day E9.5). This can be achieved by providing RA at early developmental stages via the maternal food. The doses administered are non-teratogenic, but are sufficient to rescue early cardiovascular abnormalities in Raldh2\(^{-/-}\) embryos, and to obtain mutants for analysis until E14.5 [21,22]. The minimal time frame for such a rescue is a 24 hour administration from E7.5 to 8.5 (hereafter designated as `short-term` RA supplementation). The RA supplementation can also be extended for several days, testing whether abnormalities in Raldh2\(^{-/-}\) mutants might be rescued in a non-cell autonomous manner (see below).

Mutants recovered at E12.5 (Fig. 1A,B) or E14.5 (data not shown) after short-term RA supplementation consistently showed an abnormal spinal cord. Although the neural tube had closed, it was reduced in thickness dorsally, and instead of a roof plate only a thin layer of cells was present at the dorsal midline (Fig. 1A,B, arrows). To assess if the dorsal spinal cord defects were linked to a lack of active RA signaling, we used mice harboring the RARE-hsp68-lac\(^{-}\)\(\gamma\) transgene [23], a sensitive reporter for endogenous RA activity [e.g. ref. [24]]. This transgene is strongly expressed in the dorsal-most spinal cord cells in E12.5 WT embryos (Fig. 1A,C), mirroring a conserved promoter domain regulating Raldh2 expression [25]. In Raldh2\(^{-/-}\) mutants after short-term RA supplementation, the dorsal domain of RARE-lac\(^{-}\)\(\gamma\) activity was absent, correlating with the abnormal thinning of the neuroepithelium and absence of a roof plate structure (Fig. 1B, D). A novel region of RARE-lac\(^{-}\)\(\gamma\) activity appeared in prospective interneurons, as previously described (Fig. 1B,D, white arrowheads) [19,22]. Extending the RA supplementation until E10.5 improved dorsal spinal cord morphology in Raldh2\(^{-/-}\) mutants, leading to dorsal activation of the RARE-lac\(^{-}\)\(\gamma\) reporter (Fig. 1F), yet RARE-lac\(^{-}\)\(\gamma\) activity was not as sharply restricted as in WT littermates (Fig. 1E). To further establish that RALDH2 is required for the induction of endogenous RA-responsive genes, we analyzed Rarb transcripts (Fig. 1G,H). Indeed, these were not detected in spinal cords of short-term supplemented mutants (Fig. 1H).

The specification of distinct classes of neurons initially involves diffusible signals originating from dorsal (Wnt/BMP induced) and ventral (Shh induced) patterning centers. Graded signals from these two sites induce DV-restricted homeodomain and basic helix-loop-helix (bHLH) transcription factors expression. These transcriptional targets in the mitotic progenitor zone define the dorsoventral organization of spinal cord [26,27]. Unaltered Wnt1, Wnt5a, Bmp2 and Bmp4 expression in the ectoderm and/or dorsal spinal cord neuroepithelium of E12.5 Raldh2\(^{-/-}\) mutants after short-term RA supplementation (Fig. 1I-L, insert panels, and data not shown) indicated that these dorsal inductive signals are intact. Roof plate-derived signaling is required to induce dorsal neuronal subtypes (reviewed in [12]). Further analysis of Raldh2\(^{-/-}\) mutants showed correct DV distributions of the transcription factors Math1, Ngn1, Ngn2 and Mash1 (Fig. 1 I,J, and data not shown) marking roof plate-dependent dl1-dl3 dorsal interneuron populations [28,29,30]. This confirmed intact dorsal signaling in RA-deficiency mutants. Roof plate-independent Lhx1 expression in dl4-dl6 interneurons [31] was also observed in Raldh2\(^{-/-}\) mutants (Fig. 1K,L). Our analysis thus shows that several molecular abnormalities observed at early stages of neural tube development in RA-deficient quail [5,16] or mouse models [10,32], no longer appear after mid-gestation in the rescued Raldh2\(^{-/-}\) mutants. This might be because (i) maternally administered RA could induce early regulatory events rescuing, in particular, expression of dorsal determinants, and/or (ii) other regulatory inputs eventually allowed to induce these regional determinants. Ventral patterning via Shh also appeared unaffected in E12.5 short-term rescued Raldh2\(^{-/-}\) mutants, although the most severely affected mutants showed a subtle dorsal Shh expansion (Fig. 1 M,N, insert panels). While RA has been shown to affect interneuron Dbx1 and Dlx2 expression [33], these were unaltered in the rescued Raldh2\(^{-/-}\) mutants (Fig. 1O,P, inserts, and data not shown). Reduced FGF signaling in the dorsal spinal cord of RA-deficient embryos

As RA regulates telencephalic growth [38] and neural specification by inducing FGF signaling [6], we examined whether deficiencies in this growth factor pathway might be responsible for retinoid-deficient dorsal spinal cord alterations. Immunolocalization of FGF2 revealed a striking absence in the most dorsal regions of the spinal cord in E12.5 mutants, whereas ventral levels were much less affected (Fig. 2A,B). Similar dorsal-specific reductions in pERK1/2 levels (Fig. 2C,D; Fig. S2A for western blot analysis)- indicative of intracellular FGF signaling [39]- revealed that the FGF pathway was compromised in the dorsal spinal cord. Forming spinal cord blood vessels also had strong pERK1/2 activity, again attenuated in the mutants (Fig. 2C,D). Extending RA supplementation until E10.5 restored both FGF2 and pERK expression in the dorsal spinal cord (data not shown). These observations led us to investigate FGF signaling in the neural tube of unrescued Raldh2\(^{-/-}\) embryos. While we confirmed that, at early somite stages, mutants display anteriorized Fgfb expression in the caudal neural plate [8,9], from the 8 somite-stage onwards we observed reductions in FGF signaling, assayed by p-ERK immunolocalization, in the caudal neuroepithelium and paraxial mesoderm (Fig. S2B-E). The FGF-induced intracellular inhibitors Sprouty1 (Spry1) and Spry2 (Fig. S2F,G, and data not shown) and other Fgfs (Fig 1 I and J) were also reduced in mutants (Fig. S2H,I, and data not shown). Section analysis indicated decreased FGF2 expression in dorsal regions of the neural tube of
unrescued E9.5 mutants, especially along the ventricular layer (Fig. S3A,B). p-ERK was also decreased along the ventricular layer in E9.5 mutants (Fig. S3C,D). At E10.5, in short-term rescued mutants, pERK immunoreactivity was concentrated in the ventral spinal cord, and was already attenuated in developing blood vessels (Fig. S3E,F).

Transcriptomic analysis of the spinal cord

To further identify RA-dependent pathways regulating spinal cord formation, we performed transcriptomic analysis of WT versus Aldh2−/− dissected brachial spinal cords collected at E12.5 after short-term RA rescue. Affymetrix DNA microarray analysis was performed on total RNA isolated from these samples. The validity of the screen was confirmed by reduced expression of known RA targets, such as Rarb, Hoxb8 and Hoxc6, as well as Aldh2 itself (Table 1). Analysis of transcriptional profiles using the EASE gene ontology clustering software revealed a striking number of retinoid-signaling components and homeobox transcription factors reduced in expression in the mutants (Table 1). These alterations were confirmed by ISH analysis of Olig2 expression in developing interneuron and motor neuron populations, respectively.

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Figure 1. Short-term RA-rescue of Aldh2−/− embryos reveals abnormal dorsal spinal cord development. (A,B) Transverse paraffin
sections of the brachial spinal cord of E12.5 WT (A) and Aldh2−/− (B) embryos harboring the RARE-lacZ reporter transgene, collected after short-term RA-rescue (E7.5–8.5). Embryos were X-gal stained prior to sectioning, and sections counterstained with eosin. Black arrows show defective roof plate development in the mutant. (C–F) Transverse vibratome sections of WT and Aldh2−/− embryos harboring the RARE-lacZ transgene, collected at E12.5 after short-term (C,D) or extended (E7.5–10.5; E,F) rescue, were processed for X-gal staining. These experiments confirmed the lack of RARE-lacZ activity in the dorsal spinal cord of short-term rescued mutants (B,D), whereas dorsally-restricted transgene activity was not properly restored upon extended rescue (E,F). A region of ectopic lacZ activity is seen in the middle region of the spinal cord ventricular layer, particularly under short-term rescue (white arrowheads in B,D). (G,H) ISH analysis of Rarb expression in WT and Aldh2−/− embryos collected at E11.5 after short-term RA-rescue (vibratome sections of the cervical/brachial spinal cord). (I–L) ISH analysis of Wnt1 (I,J, insets), Wnt3a (K,L, insets), Math1 (I,J, main panels), and Lbx1 (K,L, main panels) in E12.5 WT and Aldh2−/− embryos after short-term rescue. Both Wnts are expressed in cells on each side of the dorsal midline, and the pattern observed in mutants (I,J,L) reveals that both sides of the neuroepithelium did not properly merge during neural tube closure. (M–P) ISH analysis of Olig2 (M,N, main panels), Shh (M,N, insets), Pax6 (O,P, main panels) and Dbx1 (O,P, insets) in E12.5 WT and Aldh2−/− embryos after short-term rescue. Brackets and arrowheads point to abnormal downregulations in developing interneuron and motor neuron populations, respectively.

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in foregut tissues of Raldh2^{−/−} mutants at E8.5, and has been implicated in the lack of lung induction [40] and cardiac outflow tract septation [41].

Reduced Notch signaling in the RA-deficient spinal cord

FGF signaling regulates uniform induction of Notch signaling as neuronal progenitors transition to an RA-dependent differentiation status [42]. While the Notch pathway has long been implicated in neuronal differentiation [43,44,45], recent studies point to important roles in neural stem cell selection and maintenance [46,47,48]. In mutants for the Notch effectors Hes1 and Hes5, neuroepithelial cells are not properly maintained and radial glial cells prematurely differentiate into neurons [49]. In E12.5 Raldh2^{−/−} spinal cords, Delta1 transcripts (encoding a Notch ligand) were markedly reduced (Fig. 3A,B), whereas Hes1 (Fig. 3E,F) and Hes5 (Fig. 3I,J) expression were also affected. The same genes were downregulated in the early neural tube of unrescued Raldh2^{−/−} mutants at E8.5 (Fig. 3C,D,G,H,K,L), consistent with observations made in avian models [5]. This further indicates that under RA deficiency, a failure to maintain FGF and Notch signaling might affect neuronal progenitor maintenance (see below).

Disrupted dorsal root ganglia migration and survival

Retinoids may control several steps of peripheral nervous system development, by triggering neural crest cell (NCC) epithelial-mesenchymal transition, coordinating dorsal root ganglion (DRG) formation along defined rostrocaudal pathways [50], and stimulating neurite outgrowth and sympathetic neuron survival [51,52]. We examined trunk neural crest genesis and DRG formation in the rescued Raldh2^{−/−} mutants. Unaltered expression of the neural crest markers Sox10 and Pax3 at E9.5 indicated no obvious defects on the generation of NCCs in short term RA-supplemented Raldh2^{−/−} mutants (data not shown). However, postmigratory neural crest was disorganized at E10.5, as observed with several molecular markers including Sox10, Isl1 and Eya2 (Fig. 4A,C). In Raldh2^{−/−} mutants, forelimb-level NCCs migrated as partly fused sheets rather than in segmental streams. Hence, developing DRG were not properly segregated, and gangliogenesis was compromised (Fig. 4B,D). Detailed analysis on sections of E12.5 mutants with Sox10 (Fig. 4E,F), anti-neurofilaments (Fig. 4G,H), and the Notch ligand Hes1 (which marks the spinal nerves dorsal exit points, Fig. 4E,F, insets), showed that dorsal spinal nerves were disorganized and shortened, and their connections with the DRG were poorly defined (Fig. 4F,H, arrowheads).

During gestation many neurons (including some of the DRG) are eliminated by apoptosis due to a lack survival signals [53]. To examine if increased cell death accompanies DRG malformations, we performed immunodetection of the active form of caspase 3 (Act-Casp3) - a main effector of the apoptotic cascade (reviewed in [54]). Higher levels of Act-Casp3 labelling were observed in the DRG of Raldh2^{−/−} mutants, and at the level of the dorsal spinal nerve exit points (Fig. 4J, arrowheads). This increase in cell death might account for the shortened dorsal nerve tracts and overall disorganization of the dorsal nerves and DRG in the mutants. These defects were prevented by extending the RA supplementation until E10.5: in that case the Raldh2^{−/−} mutants showed similar numbers of Act-Casp3+ cells as controls (Fig. 4I,J, insets), and better organized dorsal nerve tracts (Fig. 4H, inset).

Rescued Raldh2^{−/−} mutants display an abnormal lateral motor column phenotype

Spinal motor neurons arise from common ventral progenitor domains, and are organized in columns whose identities direct correct axonal projection [55,56]. Retinoic acid generated by RALDH2 in postmitotic motor neurons acts to upregulate Lm1 expression, which is required for lateral motor columnar (LMC) subtype specification. This in turn regulates dorsal limb axonal projection pathways [17,57]. Conditional gene inactivation strategies have revealed functional contributions of RALDH2 acting both within the paraxial mesoderm and in postmitotic neurons, in order to specify LMC identity [58,59]. RA-supplemented Raldh2^{−/−} mutants display severely disorganized brachial LMCs, as evidenced by the presence of continuous Isl1-expressing ventral columns, whereas these columns are interrupted by non Isl1-expressing cells in the WT spinal cord (Fig. 5A,B, brackets). These alterations are similar, or even more severe, than those described in LMC conditional Raldh2 mutants. Furthermore, we found a severe reduction of the LMC motor pools marked by Pea3 (Fig. 5C,D), EphA4 (data not shown), Hox6 and Hox8 (Fig. 5E-H). This phenotype was partly rescued by extending the RA supplementation until E10.5, resulting in less severely reduced pools (Fig. 5D, inset). As each of the Raldh2 conditional mutants [58,59] displays a milder version of the LMC phenotype observed in the rescued Raldh2^{−/−} null mutants, our data support the idea that diffusible RA produced mesodermally and RA generated by RALDH2 in postmitotic motor neurons act synergistically to specify the LMC motor pools.
cultures, and progenitors enriched in the presence of FGF2 and EGF. Like their control littermates, Raldh2−/− spinal cords were able to generate neurospheres (Fig. 6A–D) after 8–12 days in culture. Interestingly Raldh2−/− spinal cord cultures exhibited a 25% reduction in neurosphere number at day 8 (Fig. 6E), and the resulting neurospheres were smaller in size when compared to WT controls (Fig. 6A–D).

Analysis of the ability of spinal cord-derived neurospheres to differentiate in vitro was performed to assess their lineage potential. Typically 10–12 spheres were allowed to attach to a laminin-coated coverslip. The absence FGF2 and EGF in culture stimulated this event. After 3 days, nestin positive neural progenitor cells and β-III tubulin (TuJ1 positive) differentiated neurons were detected in both WT and Raldh2−/− derived neurospheres. Notably, the nestin positive cell population was increased in the Raldh2−/− derived spheres by 20%, while differentiated neurons where decreased by half (n = 9) (Fig. 6G–O). This indicates that RA controls the fate of neuronal progenitors by promoting neuronal differentiation. The total number of cells in Raldh2−/− spheres was decreased by 50% in comparison to WT (Fig. 6F). The reduced fraction of differentiating neurons likely accounts for the progressively smaller size of mutant spheres under these conditions.

Table 1. Main categories of genes exhibiting reduced or increased expression levels from DNA microarray analysis of Raldh2−/− vs. wild-type E12.5 spinal cords (see Materials and Methods).

| Retinoic Acid Regulates Spinal Cord Development |
|-----------------------------------------------|

Table 1. Main categories of genes exhibiting reduced or increased expression levels from DNA microarray analysis of Raldh2−/− vs. wild-type E12.5 spinal cords (see Materials and Methods).

| Retinoic Signaling - Reduced | Accession # | FC (E/B) | p value |
|------------------------------|-------------|----------|---------|
| retinol binding protein 1, cellular | NM_011254 | 0.392–16.0 | 0.013163 |
| retinoic acid receptor, beta | BB266455 | 0.525–8.88 | 0.047686 |
| aldehyde dehydrogenase 1 A2 (Raldh2) | NM_009022 | 0.540–5.43 | 0.041386 |
| retinoic acid induced 2 | BB770528 | 0.669–2.14 | 0.04899 |
| SWI/SNF Smarca4 | BG064918 | 0.883–1.3 | 0.02978 |

| Homeobox Genes - Reduced |
|--------------------------|

| hox C4 | NM_013553 | 0.486–10.7 | 0.030597 |
| hox B5 | NM_008268 | 0.599–6.60 | 0.00246 |
| hox A5 | BCO11063 | 0.624–5.66 | 0.03059 |
| hox B6 | BCO16893 | 0.727–3.50 | 0.02776 |
| hox B8 | X13721 | 0.742–3.28 | 0.043881 |
| homeodomain only protein | BCO24546 | 0.807–2.46 | 0.008104 |
| hox C6 | BB440143 | 0.806–2.43 | 0.017862 |

| Structural Proteins - Reduced |
|------------------------------|

| laminin, alpha 1 | NM_008480 | 0.777–2.79 | 0.01239 |
| microtubule-associated protein 1 B | NM_008634 | 0.635–1.98 | 0.014958 |
| amyloid beta binding protein (A) 2 | BE952331 | 0.853–1.96 | 0.03334 |
| neurofilament, light polypeptide | M20480 | 0.859–1.91 | 0.023978 |
| collapsin response mediator protein 1 | AB006714 | 0.877–1.76 | 0.014808 |
| fascin-1 | BE952057 | 0.877–1.77 | 0.016426 |

| TGF-beta Induced - Increased |
|------------------------------|

| transforming growth factor-β induced | BB533460 | 1.55±3.55 | 0.020205 |
| procollagen, type I, alpha 2 | BF227507 | 1.495±3.12 | 0.013626 |
| proline 4-hydroxylase | NM_010217 | 1.45±2.8 | 0.008131 |
| procollagen, type VI, alpha 2 | AI314028 | 1.32±2.09 | 0.030724 |
| transforming growth factor, beta 2 | BA455189 | 1.26±1.82 | 0.016774 |
| tenascin C | NM_011607 | 1.15±1.41 | 0.036012 |

| Genebank accession numbers, Fold change (FC) ratios (Experimental versus Baseline, E/B) and Student’s test p values are indicated. doi:10.1371/journal.pone.0032447.t001 |

RA deficiency increases the spinal cord side population (SP) and alters its transcriptional profile

Dual-wavelength flow cytometric analysis (FACS) of cells labeled with the fluorescent DNA-binding dye Hoechst 33342 can be used to assay for the differential ability of stem cells to efflux the Hoechst dye [61,62]. Using this assay a small population of laterally shifted cells (the ‘side population’, SP) exhibits NSC potential and is inhibited in its terminal lineage commitment [63]. To investigate whether RA might regulate SP formation, brachial spinal cords were dissected at E12.5 from short-term rescued Raldh2−/− mutants and WT littermates, and assayed for SP cell numbers (n = 9 FACS experiments performed on independent pools of 10–20 spinal cords each). SP cells constituted approximately 0.6% of the sorted cell fractions from WT samples (Fig. 7A,E). Consistently, Raldh2−/− spinal cords exhibited a ~3-fold increase in SP cells, with respect to WT samples (Fig. 7B,E). The Hoechst dye efflux capacity of stem
cells is due to membrane efflux pumps of the ATP-binding cassette (ABC) transporter superfamily, including multidrug resistance 1 (MDR1) and ABCG2 [64,65]. These pumps could be blocked by verapamil in both WT and mutants (Fig. 7C,D), showing the specificity of the assay.

RNA was isolated from the sorted SP fractions, amplified and assayed by real-time quantitative PCR to test if the Raldh2 SP might be altered in its expression of pluripotency and/or NSC markers. Levels of the NSC markers Msi1 and Nestin were upregulated by about 8–9 fold, data consistent with observations of

Figure 3. Decreased Notch signaling in the retinoid-deficient spinal cord. ISH analysis of Delta1 (A–D), Hes1 (E–H) and Hes5 (I–L) was performed on transverse vibratome sections of brachial spinal cords from WT and Raldh2−/− embryos collected at E12.5 (A,B,E,F) or E11.5 (I,J) after short-term RA-rescue, and on whole-mount E8.5 unrescued embryos (C,D,G,H,K,L). Genotypes are indicated in each panel.
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Figure 4. Retinoid deficiency affects dorsal root gangliogenesis. (A–D) Whole-mount ISH analysis of Sox10 (A,B), Isl1 (C,D, main panels) and Eya2 (C,D insets) in the prospective dorsal root ganglia (drg) of E10.5 WT and Raldh2−/− embryos, collected after short-term rescue (genotypes as indicated). (E,J) ISH analysis of Sox10 (E,F, main panels) and Hrt2 (E,F, insets), anti-neurofilament (NF) staining (G,H) and activated-Caspase 3 immunodetection (I,J) on transverse vibratome sections of brachial spinal cords of E12.5 WT and Raldh2−/− embryos after short-term rescue (genotypes as indicated). Insets in H, I and J show details of a dorsal nerve root (H) and dorsal ganglia (I,J) of embryos collected at the same stage after an extended (E7.5–10.5) rescue. Red arrowheads indicate the dorsal nerve exit points, which are sites of excess apoptosis in the short-term rescued mutant (J).
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increases in nestin positive cells in the neurosphere assays. Expression of the progenitor/neurogenic markers Pax6, Olig2, and Sox2 was increased 2–6 fold in the mutant SP. The radial glia markers Bhlh and Glast, though, were dramatically reduced under RA deficiency (Fig. 8). While levels of the Med1 gene were increased 6-fold in the mutants, Abag levels were reduced by about 60% (Fig. 8). Collectively these changes indicate that the Raldh2 mutant SP is shifted towards a more pluripotent/NSC transcriptional profile, but is inhibited in its ability to generate a radial glia lineage.

Discussion

Rescue of Raldh2−/− embryos reveals abnormal spinal cord development

Raldh2 has a dynamic expression in the axial, paraxial and lateral plate mesoderm adjacent to the early embryonic neural tube. This is followed by specific expression in the developing meninges and lateral motor columns (LMC) of the spinal cord [17,66]. Initially RA sets the expression boundaries of Hoxc genes [67,68], then differentiates caudal FGF stem cell progenitors. Elaboration of DV programs of motor and interneuron differentiation [16,33,69] and induction of dorsal limb motor neuron subtypes also require RA [17,57]. Our work has shown two levels of action of RA influencing spinal cord cells during development. RA acts globally (in both neuronal and non-neuronal lineages) to maintain high levels of FGF and Notch signaling. Additionally, it promotes neuronal progenitors differentiation. In the absence of RA, progenitor cells are maintained in a multipotent state; however, reduced FGF and Notch signaling will eventually lead to a depletion of neuronal progenitors and a block of differentiation toward the radial glia lineage.

Neurite growth and cell survival is hindered in dorsal root ganglia of RA-deficiency mutants

In the peripheral nervous system RAR signaling appears to play a central role in regulating neurite extension (reviewed in [70,71]). An upregulation of Rab3 following RA treatment correlates with increased neurite outgrowth in cultured adult dorsal root ganglia (DRG), and lentiviral RARβ2 overexpression promotes regeneration of sensory axons, both in vitro and in vivo after cervical dorsal root crush injury [72,73]. RA is likely to sustain axonal growth and neuronal survival by transcriptional activation of neurotrophins and their receptors [74]. Reciprocally, the neurotrophin NGF activates Raldh2 transcription [75].

Through this regulatory loop, RA signaling will facilitate NGF and NT-3-dependent neurite outgrowth [51,52,76] and activate targets necessary for axonal elongation [77]. Upon Raldh2 loss of function there is a clear reduction in the length of spinal nerves entering the DRG, which are predictably NGF and NT-3 RA-responsive populations. Actions of NGF increasing MAPK family signaling (SAPK and ERK1/2) and preventing DRG apoptosis [78], are presumably impaired in the dorsal spinal nerves of Raldh2−/− mutants. A similar function of RA in offsetting cell death has been shown in the hind- and forebrain [79,80], potentially due to altered FGF signaling [38,81] and neurotrophin availability [53].

RA deficiency reduces FGF and Notch signaling, and neural stem cell survival

In spite of an increasing number of studies aiming to elucidate the mechanisms of regulation of neuronal subtype diversification, altogether spinal cord neurogenesis remains poorly understood. In vitro differentiation studies of embryonic stem (ES) cells are a powerful tool, offering access to molecular pathways driving neural stem cell differentiation (reviewed in refs. [82,83]). Combinatorial interactions of trophic factors appear critical in inducing neural stem cells (NSCs) toward defined lineages. Interestingly, spatial-specificity can also be induced during NSC differentiation. As an example, motor neurons induced by combined RA and Shh action express markers of cervical level spinal motor neurons [83]. There is an intrinsic temporal patterning mechanism of ES cell neurogenesis, also occurring in the developing spinal cord. Neuron generation precedes that of glial cells. A late (E12.5+ in mouse) neuron-glial switch is a general property of NSCs in all parts of the developing brain and spinal cord. The Notch signaling pathway plays a pivotal role in the maintenance of stem/progenitor cells and the neuronal to glial switch in identity [84,85,86]. This Notch-regulated glial transition is controlled by combined action of nuclear receptors COUP-TFI and COUP-TFI1 (NR2F1, NR2F2) [87], the latter being found to be a RA-activatable receptor [88].

The effects of combined reductions in FGF2/p-ERK and Notch signaling in RA-deficient spinal cords might reflect more general functions of FGF2 in increasing neurons and glia generation from cortical stem cells [89,90], and Notch signaling in first maintaining NSCs, then promoting glial fate transition [91]. Murine Hes1;Hes5 double mutants have a premature depletion of radial glial cells, leading to structural alterations of the spinal cord and fusions of the DRG [92], reminiscent of the phenotype of the short-term
rescued $Raldh2^{+/−}$ mutants. RA directs ES cells to become a uniform $RC2^{+/−}$ $Pax6^{+/−}$ radial glial lineage [93,94], indicating it may serve as a master regulator. Consistently, the fetal brain subventricular zone harbors subpopulations of RA-activated $RC2^{+/−}$, $GLAST^{+/−}$ radial glia progenitors [95]. RALDH2, by inducing meningeal or fibroblastic FGF2 production in both the fetal brain and spinal cord, may play a role in the stromal microenvironment allowing stem cell maintenance, consistent with reduced NSC survival under RA deficiency [96]; this study). The effects of RA on NSC populations appear synergistic, i.e. increasing both FGF and Notch signaling to promote NSC commitment [97].

### Perspectives for human health

We have shown that RA-deficiency abnormalities in spinal cord populations can be partly prevented by providing an extended maternal retinoid treatment, a finding which may be applicable in stem cell-based therapies. Recent studies in animal models clearly pointed out to a role of retinoids in promoting neurogenesis in the adult spinal cord [98], and stimulating axonal outgrowth after spinal injury [99]. More generally, in human RA deficiency might contribute to NSC degeneration and to some neurological diseases. Reductions in $Raldh2$ expression and RA signaling have been found in postmortem samples of patients with human amyotrophic lateral sclerosis (ALS) and Alzheimer’s disease.
ethanol during critical periods of development decreases the radial glial progenitor pool, and reduces levels of activated Notch1 and FGFR2 [109]. Impaired retinoid signaling secondary to overall vitamin A deficiency or fetal alcohol exposure, may in the long-term result in the selective depletion of radial glial NSCs. While RA treatment alone might partially restore these defects, understanding how synergistic combinations of RA, Notch, and FGF agonists boost endogenous neuroregenerative capacities may offer a plausible alternative to cell based treatments.

Materials and Methods

Ethics statement

All animals were maintained and manipulated under animal protocols reviewed by the Baylor College of Medicine and University of Texas at Austin ethics committees, which specifically approved this study (AUP 2010-00128), in strict accordance with NIH guidelines, provisions of the Guide for the Care and Use of Laboratory Animals, and the Animal Welfare Act.

In situ hybridization (ISH) and immunohistochemistry (IHC)

Raldh2-null mutants, and the maternal RA supplementation procedure, have been described previously [20,22]. All animals were maintained and manipulated under animal protocols reviewed and approved by the Baylor College of Medicine and University of Texas at Austin, in strict accordance with NIH guidelines, provisions of the Guide for the Care and Use of Laboratory Animals, and the Animal Welfare Act. Whole-mount ISH with digoxigenin-labeled riboprobes was performed as described [110], using Intavis InSituPro robots (for details, see http://www.empress.har.mrc.ac.uk/browser/, Gene Expression section). All ISH beyond E9.5 were performed on transverse (100 µM) vibratome sections of paraformaldehyde-fixed embryos. X-gal assays were performed as described [23]. Whole-mount immunolabeling with the phospho-p44/p42 Map-kinase (p-ERK1/2) antibody (Cell Signaling) or FGF2 (Santa Cruz) was performed according to the Rossant web site protocol (http://www.sickkids.ca/research/rossant/protocols/ImHis.asp). Anti-cleaved caspase-3 (Cell Signaling Technology) and mouse monoclonal anti-neurofilament (2H3, Developmental Studies Hybridoma Bank) immunohistochemistry was performed using a peroxidase-conjugated goat anti-rabbit (Pierce) secondary antibody. Fluorescence immunolabelling was performed on transverse cryosections (14 µM) of E9.5 and E10.5 paraformaldehyde-fixed embryos, with p-ERK1/2 and FGF2 primary antibodies (refs. as above) revealed with Alexa 555 secondary antibody, and nuclei stained with 4',6-diamidino-2-phenylindole (DAPI). Procedures for conventional histology can be found at http://www.empress.har.mrc.ac.uk/browser/, Histology section. Four to ten Raldh2<sup>−/−</sup> embryos were examined for all assays and at each developmental stage described hereafter. All spinal cord sections displayed are at the lower cervical/brachial level.

Embryonic spinal cord derived neurosphere culture

The dorsal region of the brachial/cervical spinal cord was dissected from individual E12.5 embryos. Neurosphere experiments were performed as described [111] with slight modifications. Briefly, cells from 4 spinal cord tissue explants were dissociated in 500 µL of non-enzymatic cell dissociation medium (Sigma) with a fired-polished Pasteur pipette. Cells were plated in 6-well plates (Nunc, non-coated) at a density of 60,000 cells/mL in DMEM:F12 medium (Invitrogen) supplemented with 40 ng/mL FGF2 (human, Peprotech), and 20 ng/mL EGF (mouse, Sigma).
Half medium was changed every 2 days. Spheres were counted under a Nikon light microscope and allowed to differentiate on laminin (Gibco)-coated 4 mm coverslips before immunostaining. Immunocytochemistry was performed according to ref. [76]. Anti-β-III-tubulin (TuJ1, Covance) and anti-Nestin (rat-401, Developmental Studies Hybridoma Bank) were used at 1/600 and 1/100 dilutions, respectively. Nuclei were stained with 0.5 μg/mL diamino-2-phenylindole (DAPI, Sigma).

Flow cytometric analysis

Lower cervical/brachial E12.5 spinal cords were dissected, washed in Hanks balanced salt solution (HBSS), and resuspended in a Liberase Blendzyme IV digestion buffer (Roche) solution for 5 minutes at 37°C. A 40-μm filtered supernatant was diluted with an equal volume of 20% serum (FBS) in Dulbecco modified Eagle medium (DMEM, Invitrogen) to stop the reaction, and tissues were pelleted and washed. After resuspension in DMEM containing 2% fetal calf serum (FCS) and 10 mM HEPES, cell concentration was adjusted to 10^6 cells/mL using DMEM. Hoechst 33342 (Sigma) was added at 5 μg/mL and the cell suspension incubated at 37°C for 60 minutes. For control experiments, the channel blocker verapamil was added at a concentration of 50 μM for 5 minutes prior to the addition of Hoechst 33342. Cell suspensions were mixed every 20 minutes, and resuspended in 4°C HBSS containing 2% FCS and 2 μg/mL propidium iodide to assay for viability. Flow cytometric analysis and fluorescence-activated cell sorting (FACS) was carried out immediately afterward using a Dako triple laser cell sorter (MoFlow, Cytomation, Fort Collins, CO). For detailed descriptions of Hoechst 33342 staining and flow cytometry, see http://www.bcm.edu/labs/goodell/index.cfm.

Real-time quantitative RT-PCR

Total RNA (100 ng) was subjected to real-time RT-PCR using SYBR Green Core Reagents (Qiagen) according to the manufacturer’s protocol. The incorporation of SYBR Green dye into the PCR products was monitored in real-time with an ABI PRISM 7700 sequence detection system (PE Applied Biosystems). Target genes were quantified relative to a reference gene, glyceraldehyde-3-phosphate dehydrogenase (Gapdh), which expression was stable in our experimental conditions. Primer sequences are available on request.
Microarray analysis

Total RNA (20 μg/t) was extracted from dissected brachial spinal cord from ten WT and Raldh2−/− mutants collected at E12.5 after short-term RA rescue. RNA was purified using the RNeasy kit (Qiagen). An Agilent low RNA input linear amplification kit was used on sorted SP populations. RNA quality was verified by using an Agilent 2100 Bioanalyzer. All subsequent reactions using Affymetrix GeneChip Mouse 430A arrays were carried out by the Baylor College of Medicine Microarray Facility as indicated at http://www.bcm.edu/microarray/, and in compliance to MIAME guidelines. Data normalization and statistical analysis was performed using dChip 1.2 (W. Wong, Harvard University, Cambridge, MA). Genes were classified into functional categories using the EASE analysis tool [http://david.abcc.ncl.ac.uk/ease/;] based on the classification scheme in Gene Ontology.

Supporting Information

Figure S1 Lack of RALDH2-mediated RA synthesis affects early neuronal determinants in the prospective spinal cord. Whole-mount ISH analysis of Pax6 (A,B), Ngn2 (C,D), Olig2 (E,F, main panels) and Irx3 (E,F, insets) in WT and unrescued Raldh2−/− embryos (genotypes as indicated in each panel). The developmental stages are 6–8 somites (A–D, insets in E,F) and E9.0 (E,F, inset in C,D). fb, forebrain; hb, hindbrain; pl, placodal cells; sc, spinal cord. (TIF)

Figure S2 Altered FGF signaling in the developing spinal cord of RA-deficient mutants. A: Western blot analysis of p-ERK levels in WT and Raldh2−/− samples. Upper panel: E9.0 (14–16 somite-stage) embryos, caudal regions, pooled, n = 15. Lower panel: E12.5 embryos collected after short-term rescue, upper cervical/brachial level spinal cords, pooled, n = 7. B-E: Whole-mount immunodetection of phosphorylated ERK1/2 (p-ERK1/2) in E8.5 (somitic stages and genotypes as indicated) WT and Raldh2−/− embryos. B,C: Profile views; D,E: details of the caudal region viewed dorsally. Brackets in B,C indicate strongly labelled extra-embryonic membranes, and in D separate domains of high labelling in somitic and caudal regions. F–I: Whole-mount in situ hybridization (ISH) analysis of Spry1 (F,G) and Fgf18 (H,I) in WT and Raldh2−/− embryos (genotypes as indicated). All embryos are viewed dorsally, and the developmental stages are 6–8 somites (F,G, and H,I, insets), and 12–14 somites (H,I, main panels). (TIF)

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