

Retinoids and Spinal Cord Development

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ABSTRACT: The role that RA plays in the development and patterning of the spinal cord is discussed. The morphogenetic process of neurulation is described in which RA plays a role because in the absence of RA signaling spina bifida results. Following neural induction, RA is involved in several patterning events in the spinal cord. It is one of the posteriorizing factors along with FGFs and Wnts and as such patterns the cervical spinal cord acting via the Hoxc transcription factors. It is involved in the induction of neural differentiation via genes such as *NeuroM*. It plays a part in patterning the dorsoventral axis of the anterior spinal cord where it interacts with FGF, Shh, and BMPs and induces an interneuronal population of neurons called V0 and V1 and a subset of motor neurons known as LMCs. To per-

form these actions RA is synthesized in the adjacent paraxial mesoderm by the enzyme RALDH2 and acts in a paracrine fashion on the developing neural tube. In the final action of RA, it begins to be synthesized within the neural tube at brachial and lumbar levels in the LMCs. Later-born neurons migrate through this RALDH2-expressing region and induce differentiation in these migrating neurons, which become a subset of LMC neurons known as LMCIs. Thus RA acts several times over in the development of the spinal cord and not on the cells in which it is synthesized, but in adjacent cells in a paracrine manner. © 2006 Wiley Periodicals, Inc. *J Neurobiol* 66: 726–738, 2006

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NEURULATION

The flat neural plate, which lies on the upper surface of the embryo and in which patterning into the forebrain, midbrain, hindbrain and spinal cord takes place [Fig. 1(A,B)] undergoes a complex series of morphogenetic movements known as neurulation to produce a neural tube that eventually lies just beneath the surface of the embryo. During neurulation the open neural plate undergoes a mediolateral narrowing, a rostrocaudal lengthening, and the neural folds elevate and fuse in the midline, while the epiblast at the lateral edges, outside the neural plate, comes to cover the newly invaginated neural tube. This tube is initially a single layer of pseudostratified epithelium,

which then proliferates rapidly, and differentiation of neurons takes place at the periphery of the expanding neural tube.

Failure to complete closure of the neural tube at the rostral end leads to anencephaly (no brain) or various degrees of cephalic tissue protruding outside the skull (exencephaly), and failure to complete closure of the neural tube at the caudal end, within the spinal cord, leads to various degrees of spina bifida. Together these defects are known as neural tube defects (NTDs) and they are very important in humans as they are the second-most common congenital defect.

It is reasonable to ask whether retinoic acid (RA) plays any role in the etiology of NTDs because the occurrence of spina bifida, anencephaly, and exencephaly is one of the well-established teratogenic effects of excess RA on a wide variety of species (Kalter and Warkany, 1959; Shenfelt, 1972; Tibbles and Wiley, 1988; Yasuda et al., 1986). The cause of this effect has been attributed to vascular damage, malfor-

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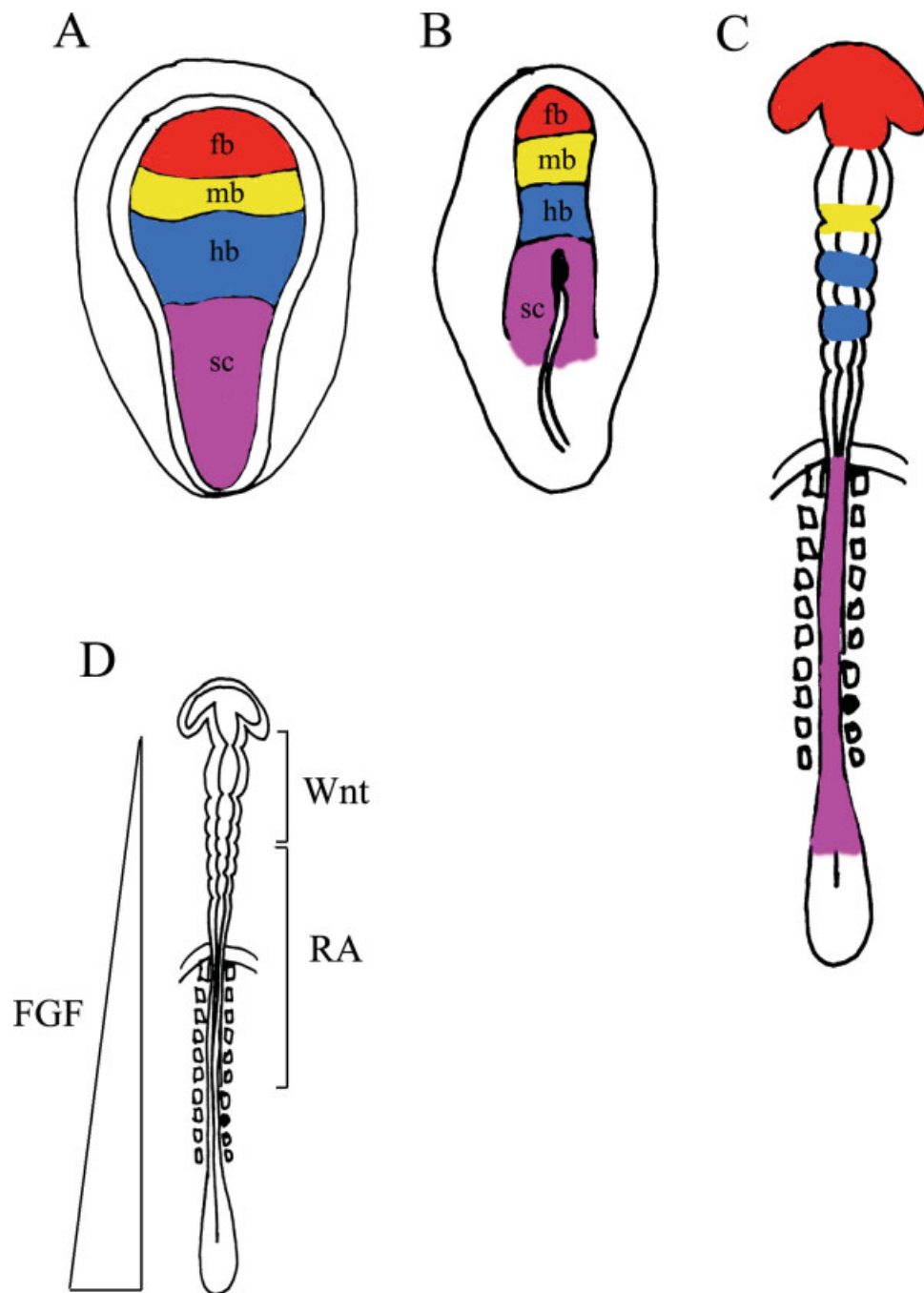


Figure 1 (A,B) Drawings of the neural plate stages of frog (A) and chick (B) embryos to show the presumptive regions. The neural plate is the colored area. In (A) the neural plate is surrounded by the neural folds and in (B) the node (black dot) is still regressing posteriorly through the presumptive spinal cord region. fb = forebrain = red; mb = midbrain = yellow; hb = hindbrain = blue; sc = spinal cord = purple. (C) The gene probes and their expression domains marked on a chick embryo that are used to identify regions of the neural plate that differentiate after various treatments. Red = *Otx2* = forebrain marker; yellow = *En1* = midbrain/hindbrain border; blue = *Krox20* = rhombomeres 3 and 5 of the hindbrain; purple = *Hoxb9* = spinal cord. (D) The regions of the nervous system that Wnts, RA, and Fgf are responsible for generating.

mation of the notochord, distortion of the neural tube, cell death, delayed posterior neuropore closure, or cell death in the tail bud (Alles and Sulik, 1990; Kapron-Bras and Trasler, 1988; Tibbles and Wiley, 1988; Padmadabhan, 1998).

However, because the levels of RA that are administered during a teratogenic dose are at least 1000-fold higher than physiological levels (Horton and Maden, 1995), it is highly likely that this type of result is not telling us anything about the role of endogenous RA in neurulation. To reveal this role we need to ask whether in the absence of RA signaling NTDs are generated. This has been performed in several ways.

Firstly, the generation of null mutant embryos for various RARs and combinations thereof has generated embryos with exencephaly. Thus there are high levels of exencephaly in *RAR α / γ* double-null mouse mutant embryos (Lohnes et al., 1994), and *RAR γ -/-* mutant embryos are resistant to the spina bifida, exencephaly, and anencephaly caused by teratogenic doses of RA (Iulianella and Lohnes, 1997; Lohnes et al., 1993). The *Cyp26A1*-/- mutant embryos mimic the effects of excess RA by exhibiting exencephaly and spina bifida (Abu-Abed et al., 2001; Sakai et al., 2001), and this phenotype is rescued in the *Cyp26A1/RAR γ* double-null mutant (Abu-Abed et al., 2003). These results suggest that endogenous RA signaling via the *RAR γ* receptor plays a role in proper closure of the neural tube during neurulation.

Secondly, the *Raldh2* null mutant embryos that have lost the major RA synthesizing enzyme in the embryo also exhibit spina bifida (Niederreither et al., 1999).

Thirdly, in the curly tail mouse mutant, which is used as a model for human NTDs, the incidence of spina bifida is reduced by the administration of low doses of RA at a specific time in development (Seller and Perkins, 1982). This effect is thought to involve the straightening of the tail bud, thereby decreasing the length of the posterior neuropore and normalizing the posterior neuropore closure (Chen et al., 1994). In this mutant the appearance of spina bifida is thought to involve the down-regulation of *RAR γ* and *RAR β* transcripts in the posterior neuropore and hindgut (Chen et al., 1995).

However, in the VAD quail model system there are no NTDs, but the neural tube fails to increase the cell proliferation rate later in development (Wilson et al., 2003). In mammals however, spina bifida is reported as a teratogenic effect of removal of RA (Walter and Warkany, 1959).

Therefore, it seems that in mammals the correct levels of RA are required for the completion of these complex morphogenetic movements known as neuro-

lation, otherwise spina bifida will result, but this does not seem to be the case in birds.

PATTERNING THE NEURAL PLATE

Patterning in the nervous system begins when the neural plate is induced from the epiblast layer, which is the uppermost layer of the three-layered embryo. This occurs by the action of the node or organizer, which, when grafted to another embryo, can induce a complete ectopic embryonic axis. The current view of the molecular basis of the induction of the neural plate is that the node secretes several molecules including noggin, follistatin, and chordin (review, Stern, 2005). These molecules are inhibitors of a family of proteins called bone morphogenetic proteins (BMPs). BMPs are produced by the cells of the epiblast, and wherever BMPs are free to act, ectoderm is induced. But where BMPs are prevented from acting by being bound in the extracellular matrix by these molecules secreted from the node, neural tissue forms. This is the so-called "default model" of neural induction (Hemmati-Brivanlou and Melton, 1997). As the neural plate develops it becomes divided up into distinct domains, namely, forebrain, midbrain, hindbrain, and spinal cord territories [Fig. 1(A,B)], which are characterized by the expression of various marker genes. These territories are induced by the underlying mesoderm and form sequentially over time after the initial induction by the node, a phenomenon that was the basis of the activation/transformation model of neural development proposed by Nieuwkoop (1952), developed from his studies on the amphibian embryo. He proposed that neural induction is a two step process. In the first step, the activation step, an activation signal from the mesoderm (chordin, follistatin, noggin) initiates neural development by inducing neural tissue of an anterior type (forebrain). In the second step, the transformation step, a mesodermal transformation signal converts this anterior neural tissue into more posterior midbrain, hindbrain, and spinal cord tissue. This transformation step could be a concentration-dependent phenomenon or could depend on different signals from different regions of the mesoderm.

With the advent of molecular markers the model has been shown to be a valid reflection of what seems to be happening. The markers typically used are *Otx2* for forebrain and midbrain, *En2* for the midbrain/hindbrain border, *Krox20* for the hindbrain, and posterior Hox genes such as *Xlhb6* or *Hoxb9* for the spinal cord [Fig. 1(C)].

Therefore, when competent epiblast is treated with these compounds that the node releases, chordin, nog-

gin, and follistatin, the whole neural plate does not form, only forebrain tissue as reflected in the expression of *Otx2* and often *En2* as well (Lamb and Harland, 1995; Hemmati-Brivanlou et al., 1994; Cox and Hemmati-Brivanlou, 1995). The transformation step is required next to generate the remaining domains from forebrain tissue and there are three molecules involved in this process, Wnts, RA, and FGFs, which are produced by the newly generated mesoderm after gastrulation. Each of these factors can induce posterior neural gene expression from this activated, anterior neural tissue. Thus, FGFs induce posterior neural gene expression (*Krox20* and *Hoxb8*) in a concentration-dependent fashion (Lamb and Harland, 1995; Kengaku and Okamoto, 1995; Cox and Hemmati-Brivanlou, 1995; Pownall et al., 1996) and are required throughout the neural plate for posterior tissue specification. Wnts induce posterior genes and suppress anterior genes leading to posteriorization of the neural tissue (Kiecker and Niehrs, 2001). The specific parts of the nervous system that Wnts (most likely Wnt8c and Wnt11) are responsible for specifying are the caudal forebrain, midbrain, and anterior hindbrain (Nordstrom et al., 2002). RA induces posterior genes (*Krox20*, *Hox* genes) and down-regulates anterior genes (*Otx2*) in noggin-induced ectoderm (Papalopulu and Kintner, 1996) and in the chick neural plate (Muhr et al., 1999), in accordance with the well-established observations of the anterior induction of posterior *Hox* genes in embryos (Conlon and Rossant, 1992). The specific parts of the nervous system that RA is responsible for specifying are the posterior hindbrain and cervical spinal cord [Fig. 1(D)].

WHERE IS THE RA AND WHAT DOES IT DO?

RA is generated from gastrulation stages onwards by RALDH2 expressed in the paraxial mesoderm (Fig. 2). It is easy to imagine how RA from the mesoderm could signal to the overlying neural plate [arrows in Fig. 2(B)] and later how RA could signal from the somites into the adjacent developing spinal cord [arrows in Fig. 2(D)]. There are no RA-synthesizing enzymes within the early neural tube (although see Mic et al., 2002; Niederreither et al., 2002). However, several studies have identified retinoids within the neural tube itself. It can be detected at high levels in the presumptive spinal cord part of the early neural tube in both the chick embryo (Maden et al., 1998) and mouse embryo (Horton and Maden, 1995) with an on/off border at the level of the first somite. Later in the d11–12 mouse spinal cord, retinol and tRA

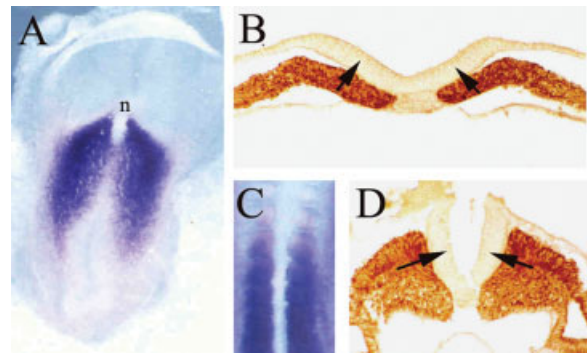


Figure 2 (A) Expression of *Raldh2* in the stage 4/5 chick embryo revealed by *in situ* hybridization showing expression in the mesoderm posterior to the node (n), either side of the primitive streak. (B) Transverse section through a stage 6 chick embryo stained with an antibody to RALDH2 showing expression in the mesoderm below the open neural plate where RA signaling occurs (arrows). (C) Expression of *Raldh2* in the stage 10 chick embryo revealed by *in situ* hybridization showing expression in the somites and not in the neural tube (central clear area). (D) Transverse section through a stage 10 chick embryo stained with an antibody to RALDH2 showing expression in the somites adjacent to the neural tube where RA signaling occurs (arrows).

(but not 9-*cis*-RA) are found throughout the cord with peak levels (four to five times higher) in the brachial and lumbar regions (Ulven et al., 2001). These regions correspond to the “hot spots” of RA synthetic activity detected by zymography (McCaffery and Drager, 1994), which may be generated by the appearance of RALDH2 within the developing motor neurons themselves (see below). The RARE-lacZ reporter mouse shows good lacZ staining in the early neural tube as well as in the somites (Mendelsohn et al., 1991; Molotkova et al., 2005; Reynolds et al., 1991; Rossant et al., 1991; Shen et al., 1992; Sirbu et al., 2005), and later peaks of activity are detected in the limb-forming regions of the neural tube (Colbert et al., 1993; Solomin et al., 1998; Mata de Urquiza et al., 1999), although in the dorsal part of the cord rather than the ventral where the RALDH +ve motor neurons are developing (see below). Thus at early stages the strong RALDH2 expression in the immediately adjacent paraxial mesoderm must be responsible for generating RA, which then diffuses into the neural tube.

There are several reasons for thinking that this paracrine action of RA is likely to be the case. Firstly, systemically administered low levels of RA enter the neural tube in preference to other embryonic tissues as revealed by the RARE-lacZ transgenic reporter mouse (Molotkova et al., 2005). Thus, this paracrine activity of paraxial mesoderm is perfectly feasible

because the neural tube can indeed take up RA from its environment. Secondly, the posteriorizing effects of these transforming molecules on anterior neural tissue are mimicked by paraxial mesoderm (Muhr et al., 1999), and there are several experiments that have shown that when cervical somites are grafted rostrally adjacent to the hindbrain the expression patterns of several *Hox* genes are altered (Grapin-Botton et al., 1997; Itasaki et al., 1996). The effects of these latter somite grafting experiments can be mimicked by the implantation of RA-soaked beads (Grapin-Botton et al., 1998) and can be inhibited by disulphiram treatment (an RA synthesis inhibitor) of the somites (Gould et al., 1998). There is also a *Raldh2* mutant zebrafish called neckless that has a neural tube defect and this can be rescued by the transplantation of wild-type mesodermal cells (Begemann et al., 2001). Thirdly, Ensini et al. (1998) have shown that when brachial and thoracic segments of the chick neural tube are exchanged or brachial and thoracic somites are exchanged then the motor neuron subtypes that develop are altered. In the former case the motor neuron subtypes in the graft are changed to resemble those appropriate to the new somitic location and in the latter case the somite grafts induce a change in motor neuron subtype appropriate to the origin of the graft.

Thus RA is generated at the right time for neural tube patterning, from gastrulation onwards, and in the paraxial mesoderm, which induces pattern in the developing neural tube (Fig. 2). But what precise role does RA play in relation to the other posteriorizing factors? In fact it is responsible for generating pattern in the posterior hindbrain (see article by Rijli) and the anterior spinal cord (see below).

RA AND ANTEROPOSTERIOR (AP) PATTERNING IN THE SPINAL CORD

The relationship between RA and the other posteriorizing factors with regard to its role in AP patterning has been answered in a study where the readout of positional information was Hoxc protein distribution in presumptive motor neurons (Liu et al., 2001). Using specific antibodies, Hoxc protein distribution could distinguish between cervical, brachial, thoracic, and lumbar regions in the chick embryo at stage 24 [Fig. 3(A)], and these patterns were stable in culture. When neural tissue fated to form the caudal cervical level was cultured from the 14–18 somite embryo for a length of time equivalent to that required for the embryo to reach stage 24 then only Hoxc6 was expressed. Tissue fated to give rise to rostral brachial levels expressed Hoxc6 and low levels of Hoxc8, tis-

sue fated to give rise to caudal brachial levels expressed Hoxc6, c8, and low levels of c9, and tissue fated to give rise to thoracic levels expressed low Hoxc6, c8 and c9. This profile is specified soon after neural plate formation, fitting in with the *Raldh2* expression in the paraxial mesoderm.

FGFs induce these Hox proteins in tissue fated to form rostral cervical cord, which would not normally express any of them. Low levels of FGF (5 $\mu\text{g/mL}$) induced c6 expression, and higher levels (25 $\mu\text{g/mL}$) expressed c6, c8, and c9. Higher levels (125 $\mu\text{g/mL}$) expressed low c6, high c8, high c9, and low c10. The highest levels (625 $\mu\text{g/mL}$) expressed low c8, high c9, and high c10. It was also found that FGF signaling ability could be enhanced by another factor, Gdf11, a member of the TGF- β family, which is expressed in the tail bud, suggesting a role for this posteriorizing factor in combination with FGF.

It was striking in these experiments that no Hoxc5-expressing cells (cervical level) were induced by FGF. When tissue fated to form rostral cervical level was cultured with cervical paraxial mesoderm and retinol many Hoxc5 cells were induced. The same result was obtained when this tissue was cultured with RA. The induction by RA was prevented by coincubation with a RAR and RXR antagonist. None of the posterior Hoxc genes were induced by RA, suggesting a specific effect on cervical spinal cord levels. When neural tissue fated to form more posterior tissue was cultured with RA then cells expressing the more posterior Hox genes were reduced in number.

On the basis of this data it was suggested that FGFs (expressed in Hensen's node) generate posterior spinal cord tissue (as reflected in Hoxc expression in presumptive motor neurons) in a concentration-dependent (higher levels might be expressed in older nodes) and/or a time-dependent (older cells have received more FGF signal) manner. Then RA from cervical paraxial mesoderm refines the general pattern by inducing cervical *Hox* genes.

Interestingly, this conclusion is supported by data from a vitamin-A-deficient quail model system. These embryos develop in the absence of RA and in the spinal cord have deficient dorsoventral (DV) patterning (see below). However, the deficiencies are only found at the cervical level of the spinal cord (Wilson et al., 2004) and not at more posterior levels.

INTEGRATION OF THE SIGNALS

It is interesting to determine how these two posteriorizing signals are integrated and how they interact

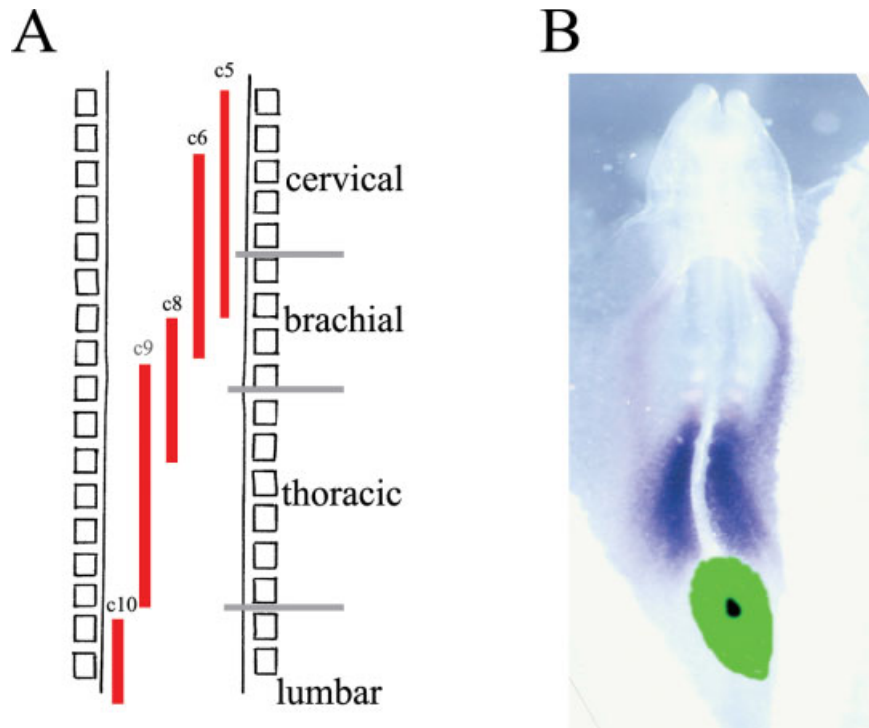


Figure 3 (A) Drawing of the expression domains of the Hoxc proteins in the developing spinal cord of the chick embryo. On the right and left are the somites divided into the four regions of the trunk (cervical, brachial, thoracic, and lumbar). In the center are the domains of Hoxc5, Hoxc6, Hoxc8, Hoxc9, and Hoxc10 proteins showing that they characterize different regions of the cord. (B) *In situ* hybridization of a stage 9 chick embryo showing the expression of *Raldh2* in the developing somites and posterior mesenchyme. Also marked is the domain of expression of *Fgf8* (green) in the caudal stem cell zone with the regressing node (black) in the center. This shows the relationship between the RA and FGF signaling regions.

during the developmental period when the spinal cord is forming. As shown in Figure 2, RALDH2 synthesizes RA in the paraxial mesoderm from gastrulation stages onwards. As the node regresses the neural tube forms in its wake and the regressing node expresses FGFs, particularly FGF8. This region has come to be called the caudal stem cell zone and recent studies have shown how the RA and FGF signals interact. As shown in Figure 3(B) the expression domain of *Fgf8* in the node, surrounding mesenchyme, and adjacent neural plate of the caudal stem cell zone and *Raldh2* in the newly segmented paraxial mesoderm are mutually exclusive and this is because they inhibit each other's expression. Thus in cultured caudal neural plate explants 9-*cis*-RA or TTNPB, a pan RAR agonist, down-regulates *Fgf8* expression and as a result promotes neural differentiation in terms of the number of *NeuroM* +ve cells (Diez del Corral et al., 2003). Conversely, in the vitamin-A-deficient quail embryo, which generates no RA, the preneural tube has stronger and prolonged ectopic *Fgf8* expression (Diez del Corral et al., 2003) as does the *Raldh2*—/—

mouse (Molotkova et al., 2005). As a result there is a drastic reduction in or absence of the expression of *NeuroM*, *Delta 1*, *Ngn1*, and *Ngn2*, and a reduced number of neurons in the spinal cord (Maden et al., 1996). Similarly, in the presence of disulphiram, the RA synthesis inhibitor, or a combination of RAR and RXR antagonists, the number of *NeuroM* +ve cells in cultured caudal neural plate explants is drastically reduced. RA is therefore required to induce neural differentiation in the newly formed neural tube and at the same time it down-regulates *Fgf* expression. This attenuation of FGF signaling is required for neuronal differentiation as ectopic FGF represses *NeuroM*, *pax6*, and *Irx3* (Bertrand et al., 2000; Diez del Corral et al., 2003).

By integrating these two patterning schemes we can therefore see that FGF signaling is required for continued growth of the neural tube, which will turn into the spinal cord, and RA signaling provided by the somites is required for the induction of neural differentiation throughout the spinal cord and the establishment of neuronal pattern in the cervical region.

This concept of RA controlling anterior spinal cord patterning is curious because *Raldh2* is expressed in all the somites as they develop, so why does the patterning aspect of RA only operate on the cervical segments? One experiment suggests that RA may not be so region specific. Treatment of the chick embryo in the rostral thoracic region with excess RA decreased the number of rostrally projecting preganglionic sympathetic neurons and treatment with disulphiram or citral in the caudal thoracic region increased the number of these neurons (Forehand et al., 1998). These authors suggested that the arrangement of sympathetic neurons in the thoracic cord is a function of segmental differences in RA levels, mediated by the adjacent somites. Thus there may be differing roles for somitic RA in different regions of the spinal cord, including both cervical and thoracic regions.

RA AND DV PATTERNING: WHICH NEURONAL TYPES DOES RA INDUCE IN THE CERVICAL REGION?

The RA signal from the cervical somites thus induces patterning via the *Hox* genes and the next consideration is which types of neuron is RA responsible for inducing? The answer has come from studies on neural plate explants and from the VAD quail model and it seems to be both interneurons and motor neurons.

The subsets of neurons that are present in the spinal cord are organized into different DV regions. In simple neuroanatomical terms, for example, the cutaneous sensory neurons form circuits in the dorsal spinal cord while visceral and motor neurons are found largely in the ventral spinal cord. Connecting these two are several interneuron populations that form distinct axonal trajectories and circuits [Fig. 4(A), right half]. These anatomical descriptions are preceded by much finer molecular descriptions of neuronal subtypes that have gradually been accumulated as more genes and proteins have been characterized. Thus the early neural tube is now divided into six dorsal populations (dl1–dl6), the interneurons and ventral motor neurons are divided into five groups (V0–V3 and MN), and there is a group of cells at the dorsal midline called the dorsal roof plate and one at the ventral midline called the ventral floor plate [Fig. 4(A), left half]. Thus, 13 populations of cells can be identified by their combinatorial expression of transcription factors.

As well as RA there are two other important signaling molecules that pattern the spinal cord in the DV axis, Shh and BMPs [Fig. 4(B)]. Shh, generated ventrally in the notochord and floor plate, acts in a

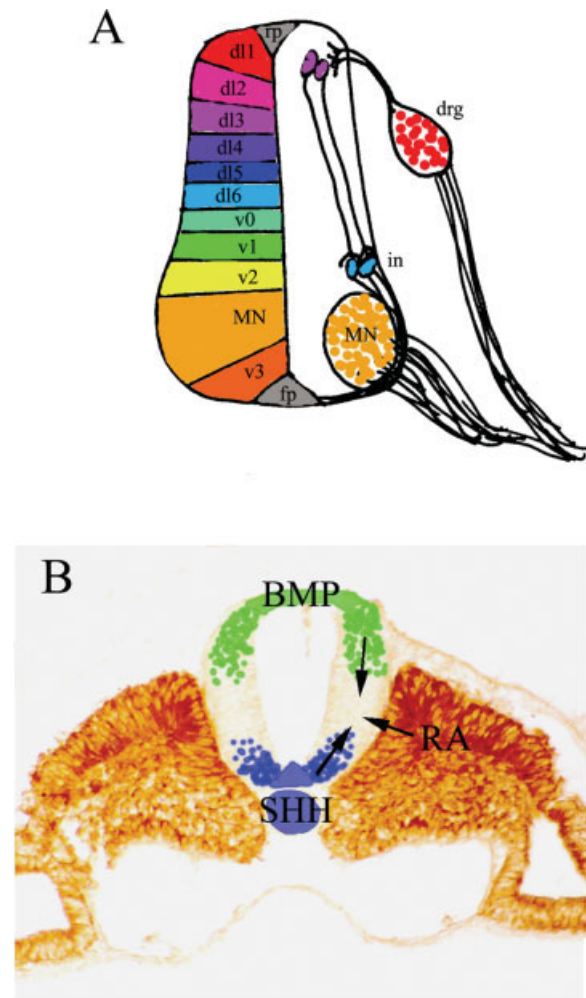


Figure 4 (A) Drawing of a spinal cord showing on the left half the 11 neuronal progenitor domains that are recognized in the cord as characterized by the expression of various gene and protein markers. The dorsal sensory domains are dl1–dl6, the ventral interneuron domains are v0–v2, the motor neurons are MN, and a further ventral domain is v3. The roof plate (rp) and floor plate (fp) are marked accordingly. On the right half is a simple drawing of where sensory neurons (red) from the dorsal root ganglion (drg) connect, where the interneurons connect (purple and blue), and where the motor neurons are located (gold). (B) Transverse section through a stage 14 chick embryo showing the three signaling molecules that pattern the dorsoventral axis. In brown is shown by immunocytochemistry the expression of RALDH2 in the somites from which RA signals arise. In the neural tube the expression and diffusion of BMP proteins from the dorsal region (roof plate) are marked in green and expression and diffusion of Shh protein from the ventral region (floor plate) is marked in blue.

concentration-dependent manner and at high concentrations (close to the floor plate) induces motor neurons (V3 and MN) and at lower concentrations (fur-

ther away from the floor plate) induces several classes of ventral interneurons (V0–V3) (Ericsson et al., 1996, 1997; Marti et al., 1995; Roelink et al., 1995). This induction occurs in a concentration-dependent manner *in vitro*, ectopic expression of Shh induces ectopic floor plate and motor neurons, the inhibition of Shh signaling with blocking antibodies stops motor neuron differentiation, and in the *Shh*^{−/−} mutant mouse no motor neurons develop (Litlington and Chiang, 2000). At the dorsal pole of the spinal cord BMPs are produced in the roof plate and these proteins are responsible for generating dorsal cell types (dl1–6) in a concentration-dependent fashion, equivalent to Shh at the ventral pole (Lee and Jessell, 1999; Liem et al., 1995, 1997; Helms and Johnson, 2003) [Fig. 4(B)]. This concept has been developed in precisely the same way as for Shh ventrally. For example, when the roof plate is genetically ablated, several of these dorsal populations do not form (Lee et al., 2000).

RA, arising from the adjacent somites [Fig. 4(B)] fits into this scheme by being responsible, at these stages, for generating certain interneuronal and ventral neuronal populations. Thus when intermediate neural plate explants are cultured with RA, *Dbx1*, *Dbx2*, *Irx3*, *Pax5*, *Evx1/2*, and *En1* are induced and *Pax7* is repressed, that is, V0 and V1 interneuron progenitors are generated (Novitsch et al., 2003; Pierani et al., 1999). Later in development excess RA increases the proportion of caudally projecting interneurons, thus affecting not only their specification but also their axonal orientation (Shiga et al., 1995). That this activity does indeed arise from the paraxial mesoderm was shown by incubating the neural plate with mesoderm and then preventing the mesodermal induction of these neuronal progenitors with RAR and RXR antagonists. Electroporation of a dominant-negative RA receptor α into the neural tube to inhibit RA signaling reduces the expression of *Pax6*, *Irx3*, *Dbx1*, and *Dbx2* (Novitsch et al., 2003). Similarly, in the VAD quail and the *Raldh2*^{−/−} mouse the expression of the ventral genes *Pax6*, *Irx3*, *Nkx6.2*, *Olig2*, and *En1* is down-regulated (Diez del Corral et al., 2003; Molotkova et al., 2005; Wilson et al., 2004). The protein products of the genes that RA induces are transcription factors known as Class I homeodomain proteins.

RA could act directly on the promoters of these progenitor genes to induce their expression and this may well be the case, but RA also needs to counteract the negative forces of the other two signals in DV patterning, Shh and BMPs [Fig. 4(B)]. Thus, high levels of BMPs repress *Dbx1* and *Dbx2* and high levels of Shh also repress these two genes (Pierani et al.,

1999). Therefore it is possible that RA keeps BMP and Shh expression at bay either by repressing *Shh* and *Bmp* expression or by repressing the genes that they induce at low levels. To test this one can examine progenitor gene expression domains in the absence of RA and this has been done in the VAD quail. In this case there is a dorsal expansion in the domain of *Shh* expression and other ventral genes in accordance with this idea, but there is a regression of dorsal genes such as *Pax3*, *Pax7*, *Wnt1*, *Wnt3a*, *Msx2*, *Bmp4*, and *Bmp7*, which does not support this idea (Wilson et al., 2004). Indeed, RA does not repress *Bmp4* expression in neural plate explants *in vitro* whereas Shh does (Pierani et al., 1999). Therefore RA seems to act on these V0 and V1 interneuron progenitors possibly directly on their promoters, but certainly by repressing *Shh* to allow *Dbx1* and *Dbx2* expression by a derepression mechanism.

Another gene expressed ventrally that RA plays a part in inducing in combination with Shh is *Olig2*, whose expression marks a motor neuron progenitor state. In this domain two other genes, *Nkx6* and *Pax6*, need to be expressed and they act as repressors to prevent the expression of transcription factor capable of repressing *Olig2* (Briscoe et al., 2000; Lu et al., 2002; Novitsch et al., 2001; Sander et al., 2000). *Olig2* itself acts as a transcriptional repressor to direct the expression of downstream homeodomain regulators of motor neuron identity via Mnx (Mnr2 and Hb9) and LIM (Isl1/2 and Lim3) proteins (Mizuguchi et al., 2001; Novitsch et al., 2001; Rowitch et al., 2002; Scardagli et al., 2001; William et al., 2003). Joint exposure of neural plate cells to Shh and RA induces high levels of *Olig2* expression (Novitsch et al., 2003). Conversely, electroporation of a dominant negative RAR α receptor into neural plate cells prevented the expression of *Olig2* +ve even in the presence of Shh. When electroporated *in vivo* into the neural tube this dominant negative construct down-regulated *Pax6* expression and virtually eliminated *Olig2* cells. In the absence of RA in the VAD quail, *Pax6*, *Nkx6.1*, *Hb9*, *Mnr2*, and *Olig2* are down-regulated (Diez del Corral et al., 2003; Wilson et al., 2004) and the number of Islet1 +ve ventral motor neurons is depleted as a result (Maden et al., 1996). The same result is found in the *Raldh2*^{−/−} mutant mouse (Novitsch et al., 2003).

Thus, after Shh from the floor plate has established the general conditions for motor neuron differentiation, RA signaling from the somites sets in train a sequence of further differentiation steps, which are summarized in Figure 5. These subsequent steps from *Olig2* expression leading to motor neuron differentiation are also down-regulated in the absence of RA or

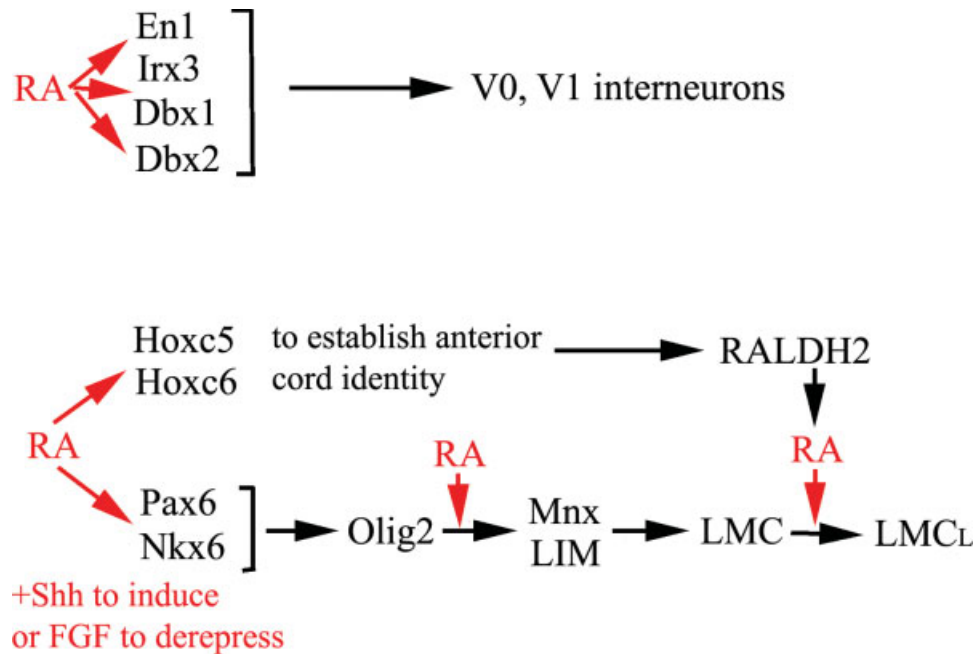


Figure 5 The gene pathways that are involved in the production of V0 and V1 interneurons and in motor neurons and the points of action of RA.

RA signaling, but not because RA has kicked off the process and it continues under its own momentum. Instead, continual RA signaling is required. This was shown by coelectroporating the dominant negative *RARα* construct and *Olig2*, whereupon *Olig2*-expressing cells still did not progress to express *Mnx*, *Lim3*, or *Isl1/2* (Novitsch et al., 2003). Not all motor neurons are dependent upon RA for their differentiation, however, only a group known as the lateral motor column (LMC), which is why there are still some *Isl1* +ve motor neurons present in the VAD quail spinal cord.

But this is not the end of the motor neuron story, because RA continues to be required for the differentiation of a subset of LMCs, and in these later steps the source of RA is provided from within the neural tube and not from the somites.

SPECIFICATION OF MOTOR NEURONAL SUBTYPE

Up to this stage RA has been synthesized outside the neural tube in the paraxial mesoderm (Fig. 2), but from stage 19 in the chick and day 12.5 in the mouse RALDH2 begins to be expressed in the ventral motor neurons themselves within the spinal cord [Fig. 6(A)]. Furthermore, RALDH2 is only expressed at brachial

and lumbar levels from where the limbs will appear and become innervated, and not in between in the thoracic region (Niederreither et al., 1997; Sockanathan and Jessell, 1998; Zhao et al., 1996). These regions of the spinal cord opposite to where the limb buds grow out had previously been identified as “hot spots” of RA synthesis (McCaffery and Drager, 1994), endogenous retinoids (Ulven et al., 2001), and LacZ activity (Colbert et al., 1993; Solomin et al., 1998; Mata de Urquiza et al., 1999), and this endogenous synthesis of RA is clearly the reason why. Interestingly, RALDH2 expression at brachial levels can itself be induced by *Hoxc6* as ectopic *Hoxc6* expression at thoracic levels induces RALDH2 (Dasen et al., 2003). This is a fascinating reverse reiteration of the original induction of anterior *Hoxc* genes by RA from the somites, as described above.

There are several types of motor neurons arranged in discrete columns along the spinal cord and which are identified by their location and innervation patterns (Landmesser, 2001). There is a median motor column (MMC), which is divided into a medial group of neurons innervating axial muscles and a lateral group innervating the body wall muscles. These are spread all along the spinal cord. At limb levels—brachial and lumbar—there are LMC neurons that innervate limb muscles, and these are divided into a medial group (LMCM), which project to ventral muscles, and a lateral group (LMCL), which project

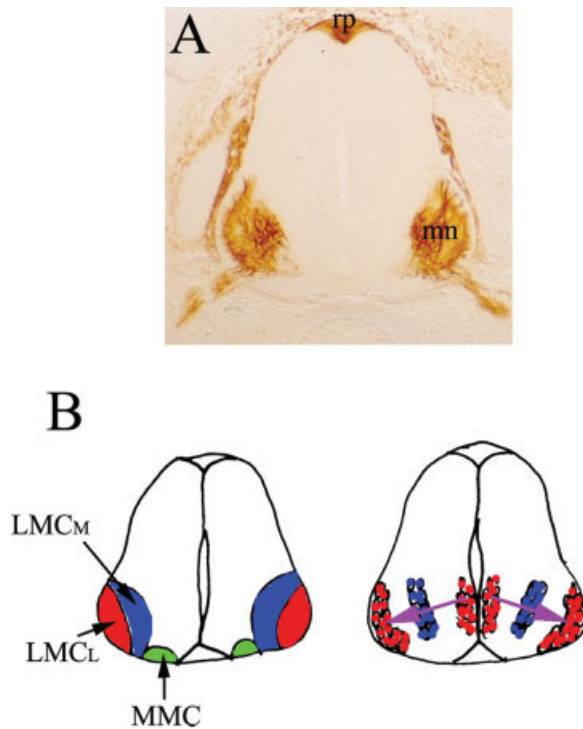


Figure 6 (A) Transverse section through a stage 28 chick spinal cord at the brachial (forelimb level) stained with an antibody to RALDH2 showing that the motor neurons (mn) are expressing this enzyme as well as the roof plate (rp) and the cells surrounding the cord, which will become the meninges. (B) Left side: drawing of a brachial level chick spinal cord showing the location of the median motor column (MMC = green), the lateral part of the lateral motor column (LMCL = red), and the medial part of the lateral motor column (LMCM = blue). Right side: drawing to show the development of the LMCLs. They are born at the ventricular surface (red cells at the midline) and migrate in the direction of the purple arrows through LMCs that have already differentiated and are expressing *Raldh2* (blue rows of cells). In so doing they become induced by the RA that these blue cells release and differentiate into LMCLs when they have reached their final resting place on the periphery of the cord (outermost red cells).

to dorsal muscles [Fig. 6(B)]. The coincidence of RA “hot spots”, RALDH2 expression in motor neurons at limb levels, and LMCs appearing at limb levels suggests a role for RA here and this is indeed the case. However, it is interesting to note that RARE-lacZ activity is seen in dorsal regions of the cord whereas RALDH2 is expressed ventrally.

LMCL neurons are born later than LMCM neurons and they are located at the lateral extremity of the ventral horn. Because neurons are born towards the ventricular surface presumptive LMCLs have to migrate through earlier born LMCs [Fig. 6(C)]. It is

the earlier-born LMCs that express *Raldh2* and synthesize RA, and it seems that this RA induces the LMCL phenotype in these later-born neurons as they migrate through the region of high RA synthesis. Excess RA increases the number of Islet 1 +ve neurons in cultured thoracic (nonlimb level) neural tube, and brachial (limb level) neural tubes have decreased numbers of LMCLs when cultured in the presence of RA receptor antagonists, which inhibit RA signaling (Sockanathan and Jessell, 1998). When thoracic (nonlimb) neural tube was transfected with *Raldh2* many LMCLs were induced, but they were not the cells that had been transfected, instead they were adjacent to them. The electroporation of a dominant negative RAR α into the brachial cord inhibits LMCL production and reduces the projection of motor neurons into the limb (Sockanathan et al., 2003). Instead, the inhibited neurons become autonomic motor neurons of the Column of Terni (CT) and lateral MMC neurons, both characteristic features of a thoracic cord. Conversely, electroporation of a constitutively active receptor into the thoracic cord perturbs CT and lateral MMC differentiation and they undergo apoptosis. Thus it seems that RA signaling is crucial to the development of the appropriate brachial level motor neuron complement.

SUMMARY

RA is required time and time again during nervous system development for a series of inductive events. Firstly, it acts as a posteriorizing factor during neural induction along with Wnts and FGFs to pattern the anterior spinal cord. This inductive event occurs via the *Hoxc* genes. RA is next required for the induction of a subset of interneurons known as V0 and V1 and which are characterized by the expression of Dbx1, Dbx2, and En1 proteins. RA is then required for the induction of neural differentiation throughout the spinal cord via the gene *NeuroM*. RA is next required in collaboration with Shh to set motor neuron differentiation in progress via the gene *Olig2* towards the production of LMC neurons. To perform these first four inductive events RA is synthesized in the adjacent mesoderm/somites and RA diffuses into the overlying neural plate/neural tube in a paracrine fashion. Subsequent stages of motor neuron differentiation whereby a subset of later-born motor neurons is induced to differentiate into LMCLs after migrating through a RA signaling region consisting of earlier-born LMCs are also paracrine events. Thus the role of RA in the development of the spinal cord reveals the inductive na-

ture of this extracellular signaling molecule in neuronal differentiation and patterning.

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