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Review

The mechanisms of dorsoventral patterning in the vertebrate neural tube

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Abstract

We describe the essential features of and the molecules involved in dorsoventral (DV) patterning in the neural tube. The neural tube is, from its very outset, patterned in this axis as there is a roof plate, floor plate, and differing numbers and types of neuroblasts. These neuroblasts develop into different types of neurons which express a different range of marker genes. Early embryological experiments identified the notochord and the somites as being responsible for the DV patterning of the neural tube and we now know that 4 signaling molecules are involved and are generated by these surrounding structures. Fibroblast growth factors (FGFs) are produced by the caudal mesoderm and must be down-regulated before neural differentiation can occur. Retinoic acid (RA) is produced by the paraxial mesoderm and is an inducer of neural differentiation and patterning and is responsible for down-regulating FGF. Sonic hedgehog (Shh) is produced by the notochord and floor plate and is responsible for inducing ventral neural cell types in a concentration-dependent manner. Bone morphogenetic proteins (BMPs) are produced by the roof plate and are responsible for inducing dorsal neural cell types in a concentration-dependent manner. Subsequently, RA is used twice more. Once from the somites for motor neuron differentiation and secondly RA is used to define the motor neuron subtypes, but in the latter case it is generated within the neural tube from differentiating motor neurons rather than from outside. These 4 signaling molecules also interact with each other, generally in a repressive fashion, and DV patterning shows how complex these interactions can be.
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Keywords: Dorsoventral patterning; Neural tube; Retinoic acid; FGF; Shh; BMP

Introduction

Even without the benefit of modern molecular markers, it is obvious that the developing spinal cord is dorsoventrally organized (Fig. 1A). The roof plate is a thin and narrow region composed of a single row of cells whose nuclei are located at the periphery and there is an absence of neuroblasts since these cells will become radial glia. The floor plate at the ventral pole is similarly organized and structured, but slightly wider in extent. The region between these two extremes is densely packed with neuroblasts, and towards the ventral region, the motor neurons which generate a slight bulge in the cord are less densely packed. The alar (dorsal) and basal (ventral) plates have different proliferation rates whereas the roof plate and floor plate have no or very low proliferative activity.

Once these neuroblasts have differentiated into mature neurons, they also show dorsoventral (DV) organization within the spinal cord because functionally distinct neurons are anatomically segregated. Thus cutaneous sensory neurons form circuits in the dorsal spinal cord while visceral and motor neurons are found largely in the ventral spinal cord (Brown, 1981; Jessell, 2000). Connecting these two are several interneuron populations that form distinct axonal trajectories and circuits (Fig. 1B). There are now many molecular markers of neuronal populations in the spinal cord which have been extensively used for studies of DV patterning and have resulted in the identification of further sub-groupings. The dorsal neurons are now subdivided into 6 groups, the interneurons and ventral neurons are divided into 5 groups, and each domain is characterized by distinct gene expression markers, as shown in Fig. 1C. This is the

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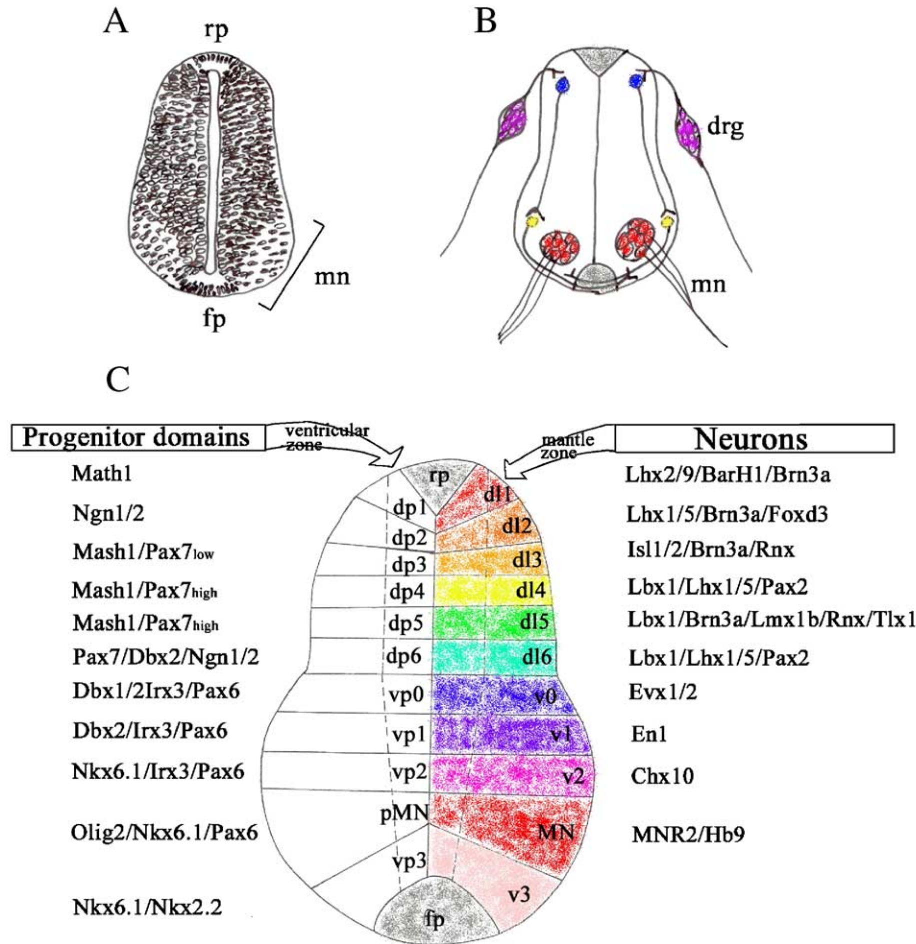

Fig. 1. (A) Drawing of an early neural tube with the cells drawn in to show that there is considerable dorsoventrality even without any modern molecular markers. At the dorsal pole is the roof plate (rp), a single line of cells with the nuclei at the margin, and at the ventral pole is the floor plate (fp) where the cells are similarly arranged. In the body of the neural tube, there are many densely packed neuroblasts, but towards the ventral region, there is a swelling where the neuroblasts are not so densely packed and these are the presumptive motor neurons (mn). (B) The neural tube in A differentiates into many different neuronal types, the major ones are shown here. Sensory neurons from the dorsal root ganglia (drg, purple) enter the dorsal cord and synapse there. In the ventral region, the motor neurons differentiate (mn, red). In between these two are various types of interneurons with axon trajectories which connect the sensory and motor regions (blue neuron) or connect one side of the cord to the other (yellow neuron). (C) Diagram to show the regionalization of the 6 types of dorsal neurons (dl1–dl6) and the 5 types of ventral neurons (v0–v3 + mn) in the developing neural tube. On the left are the gene and protein markers which are used to identify the progenitors domains (in the ventricular region close to the midline) of these different DV regions. On the right are the gene and protein markers which are used to identify the neuronal types (in the mantle region where neurons differentiate).

essence of DV patterning—how do these groups arise in the correct position?

Neural induction and caudal regression

The generation of a coarse DV pattern in the developing spinal cord takes place after neural induction. During this induction, the neural-inducing signals, currently thought to be fibroblast growth factors (FGFs), are released by the organizer and these counteract bone morphogenetic protein (BMP) signaling from the non-neural ectoderm to generate the neural plate (Munoz-Sanjuan and Brivanlou, 2002; Stern, 2001; Wilson and Edlund, 2001). The early neural plate is initially rostral (forebrain) in character and more caudal regions (midbrain, hindbrain, spinal cord) form as a result of the caudal regression of the organizer (Hensen's node in the chick). During this rostrocaudal extension of the neural plate and elaboration of the pattern in this axis, DV patterning is simultaneously taking place.

What are the developmental events that result in the establishment of early DV pattern?

The surrounding mesodermal structures generate DV differences in the neural tube

As a result of studies on amphibians from the 1920s onwards, it became clear that the surrounding/underlying mesoderm was the major determinant of the dorsoventral structure of the neural tube rather than it being due to any intrinsic self-organizing capacity (review Holtfreter

and Hamburger, 1955). In the central midline of the developing embryo is a mesodermal structure, the notochord (Fig. 2A), and laterally to the neural tube is the mesoderm, which segments into the somites. In the absence of the notochord, the characteristically thin floor plate is missing and a thick mass forms ventrally instead (Fig. 2B). The notochord, together with unilaterally located somites produces an asymmetrical cord (Fig. 2C) thickened on one side only. In the presence of two notochords, the neural tube forms two floor plates. If the two notochords are unequal in size, the smaller notochord induces less of a floor plate response from the neural tube than the larger one and the floor plate response fails entirely if mesenchyme is inserted between the notochord and neural tube.

The same principles were reinforced in subsequent experiments on chick embryos from the 1950s onwards. For example, a chick with a duplication of the anterior end had a triangular-shaped neural tube with two floor plates (Watterson et al., 1955) (Fig. 2D). Again, if the notochords are unequal in size, an unequal response in the neural tube is elicited and it was also clear that contact was essential for the formation of the floor plate. In the absence of candidate-inducing molecules to provide a molecular explanation, the opposing effects of the notochord and somites on the neural tube were said to lie in proliferation. Thus, the notochord

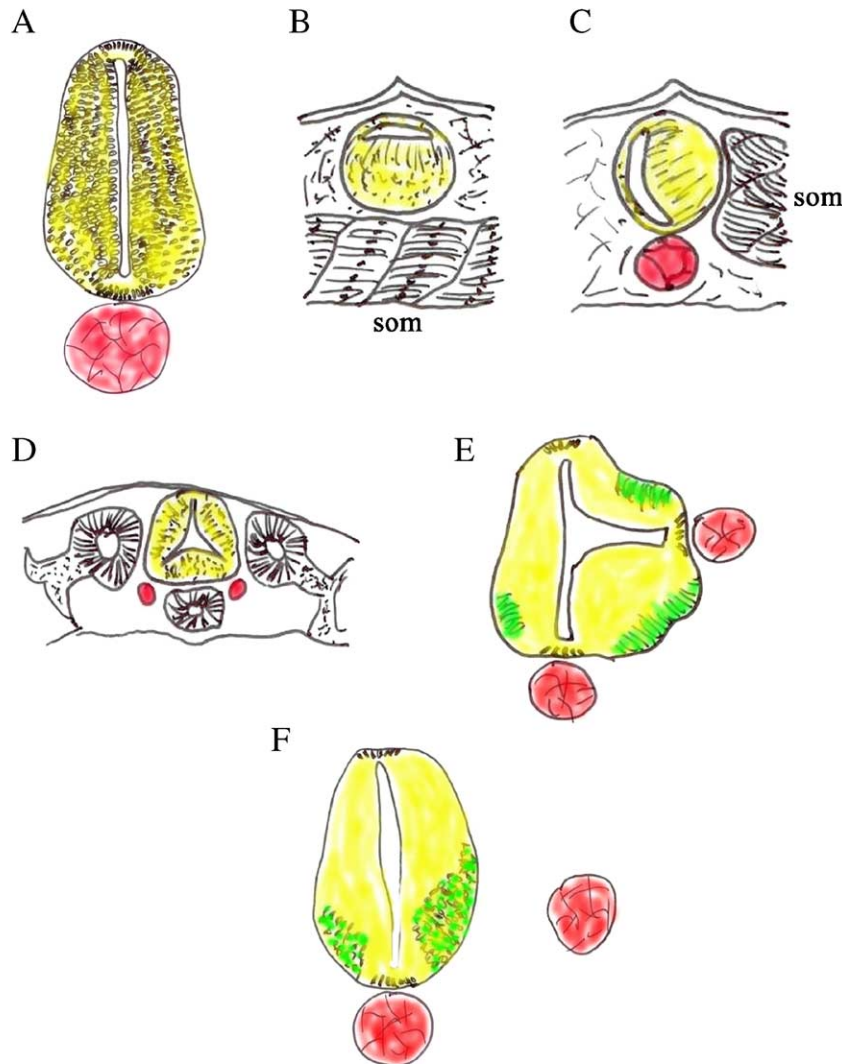

Fig. 2. (A) Drawing of the normal chick embryo neural tube (yellow) showing the location of the notochord (red) beneath the floor plate. (B) The effect of removing the notochord in a frog embryo. The floor plate is absent because the whole of the ventral neural tube proliferates under the influence of the somites (som) which spread below the neural tube. (C) The effect of removing the somites on the left of the neural tube leaving only the somites on the right (som). On the right, the neural tube is thicker than usual, and on the left, it is thinner. (D) A section through a chick embryo with a duplicated anterior end with 2 notochords (red). Two floor plates are present in the triangular-shaped neural tube and three blocks of somites. From Watterson et al. (1995). (E) The effect of grafting an extra notochord adjacent to the neural tube in a chick embryo. The extra one (on the right) has induced an ectopic floor plate and extra motor neurons which stain with AChE (green). (F) The effect of grafting an extra notochord some distance from the neural tube in a chick embryo. Here, no floor plate has been induced, but extra neuroblasts have proliferated (green). E and F are drawings from van Straaten and Drukker (1987) and van Straaten et al. (1988).

induced a local inhibition of mitosis to make the floor plate thin, and if it is removed, there is considerable overgrowth of the neural tube (Burda, 1968). Conversely, the somites induced a local stimulation of mitosis to make the lateral walls thick (Watterson, 1965). Interestingly, the wheel has now come a full circle in that recent results have revealed that FGF and *sonic hedgehog* (*Shh*) operate on the cell cycle regulators cyclin D1 and cyclin D2 (Lobjois et al., 2004) and that the roof plate inducing gene *Lmx1a* causes cells that express it to withdraw from the cell cycle (Timmer et al., 2002).

More recent chick work involving the grafting of an extra notochord (van Straaten and Drukker, 1987; van Straaten et al., 1985a,b, 1988) confirmed the inductive effect on the floor plate, but added an extra dimension. This was that neuroblasts (presumptive motor neurons) can also be induced, i.e., proliferation can be induced, but at a greater distance from the notochord than that required for floor plate induction (the latter being 25 μm). Therefore, the notochord is capable of inducing opposing effects depending upon the 'concentration'—at high levels (close to the neural tube), it inhibits proliferation and induces floor plate (Fig. 2E), and at low levels (further away from the neural tube), it stimulates proliferation and induces neuroblasts (motor neurons) (Fig. 2F).

We now know a considerable amount about the molecules involved in DV patterning and the understanding of their action relies to a significant degree on the foundation provided by these early embryological experiments.

Establishing the basics of DV patterning—the 4-signal model

As the node regresses (see above) and continues to produce FGFs, in particular FGF8, a caudal stem zone is established (Fig. 3). Rostral to this zone, neural differentiation commences within the newly generated neural plate and simultaneously rostrocaudal (RC) and DV signaling systems operate to generate pattern prior to neurulation and the closure of the neural tube (Colas and Schoenwolf, 2001; Diez del Corral and Storey, 2004). The initial DV patterning is set up by the action of 4 extracellular signaling molecules. Firstly, FGF8 within the neural cells which have just left the caudal stem zone is switched off. Secondly, retinoic acid (RA) is generated in the paraxial mesoderm by the enzyme retinaldehyde dehydrogenase 2 (RALDH2). Thirdly, sonic hedgehog (Shh) is expressed by the notochord and induced in the ventral floor plate. Fourthly, bone morphogenetic proteins (BMPs) are expressed by the overlying dorsal ectoderm and roof plate. As the embryological experiments described above established, patterning in the neural tube is thus a result of external influences and not a self-organizing capacity (Fig. 3).

Switching off FGF8

It has been established that the maintenance of caudal progenitors in this stem zone requires FGF signaling while the attenuation of FGF is necessary for neuronal and

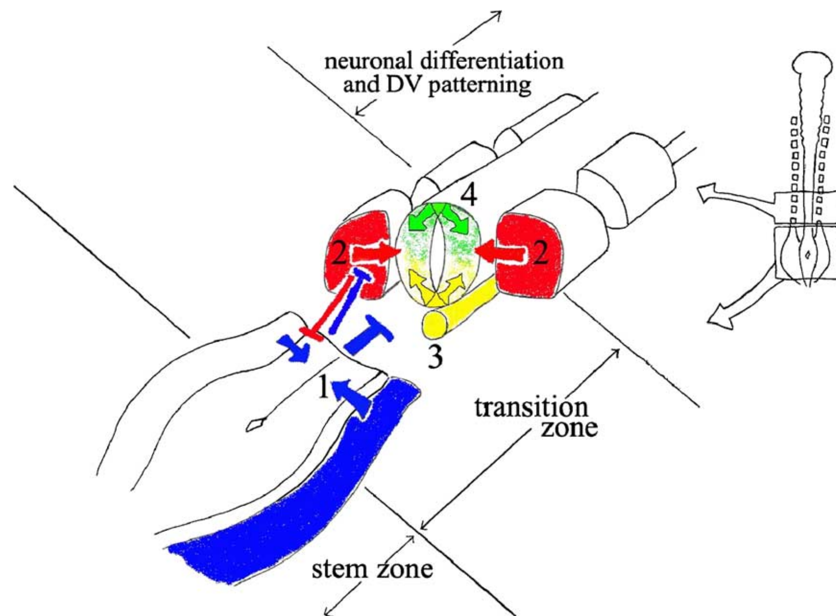

Fig. 3. Summary diagram of the posterior end of the embryo where DV patterning is taking place. In the stem zone, FGFs from the underlying mesoderm (blue) prevent neural differentiation in the overlying neural plate (signal 1). In the transition zone, the notochord differentiates and starts to express Shh (yellow, signal 3). The somites differentiate and start to express RALDH2 (red) which synthesizes RA (signal 2). BMPs start to be produced form the roof plate (green, signal 4). In the neuronal differentiation and DV patterning zone, RA antagonizes FGF and vice versa, RA induces a specific set of genes in the neural tube (red arrow), Shh is induced in the floor plate and spreads dorsally in a concentration gradient (yellow arrow), and BMPs spread ventrally in a concentration gradient (green arrow).

mesodermal differentiation (Diez del Corral et al., 2002). *Cash4*, *Sox1*, *Delta 1*, and *bra* are gene markers of this caudal zone and their expression is maintained by FGF. Over-expression of a dominant-negative fibroblast growth factor receptor (FGFR) or application of the FGFR antagonist SU5402 induces movement of cells out of the stem zone into the neural tube where they can differentiate, and removal of the presomitic mesoderm, the source of FGF, results in the down-regulation of these markers (Henrique et al., 1997; Spann et al., 1994; Storey et al., 1998). An early marker of neural differentiation is *NeuroM* and FGF inhibits its expression. *NeuroM* begins expression in the embryonic neural tube just where the somites begin to differentiate and somitic tissue promotes and is required for neuronal differentiation. Furthermore, somitic tissue inhibits *Fgf8* expression in the caudal zone. A mutual antagonism is thus established between the caudal zone (maintaining cell cycling, inhibiting neuronal differentiation) and the somites (down-regulating FGF8 and promoting neuronal differentiation) (Diez del Corral et al., 2002).

RA is the somite factor

The somites synthesize several active retinoids (Maden et al., 1998), and from gastrulation onwards, paraxial mesoderm expresses high levels of the RA synthesizing enzyme RALDH2 (Berggren et al., 1999; Blentic et al., 2003; Niederreither et al., 1997; Swindell et al., 1999). Recent studies involving the manipulation of RA signaling have shown that this RA is the factor in the somites which inhibits FGF signaling in the neuroepithelium (Fig. 3) and paraxial mesoderm as well as promoting differentiation in the neuroepithelium (Diez del Corral et al., 2003; Novitch et al., 2003). RA mimics the ability of the somites to induce *NeuroM* expression in caudal neural plate explants. The inhibitor of RA synthesis, disulphiram, or the use of retinoic acid receptor antagonists which block RA signaling, prevents the increase in *NeuroM*. In quail embryos which have no RA present, there is a vast reduction in the number of neurons (Maden et al., 1996) and the expressions of *NeuroM*, *Delta 1*, and *Neurogenin1* and *2* are all depleted or absent in the posterior neural tube (Diez del Corral et al., 2003). RA down-regulates the expression of *Fgf8* in caudal explants, and in the RA free quail embryo or *Raldh2* −/− mutant mouse (Molotkova et al., 2005), *Fgf8* expression is prolonged and stronger in the preneural tube. Conversely, FGF8 down-regulates *Raldh2* in the paraxial mesoderm, demonstrating the mutual repressive interactions between these two extracellular molecules (Fig. 4).

Genes such as *NeuroM* are pan-neural and therefore not specifically concerned with DV patterning. When this RA/FGF antagonism was investigated with DV gene markers, it became apparent that it was ventral neural genes which were the target of RA and FGFs. Thus, in the RA free quail embryo, the expressions of the ventral genes *Pax6*, *Irx3*, *Nkx6.2*, *Olig2*, and *En-1* (Fig. 1C) are all down-regulated (Diez del Corral et al., 2003; Molotkova et al., 2005; Wilson et al., 2004) and the number of Islet1+ ventral motor neurons is depleted (Maden et al., 1996). Electroporation of a dominant-negative retinoic acid receptor into the neural tube to inhibit RA signaling reduces the expression of *Pax6*, *Irx3*, *Dbx1*, and *Dbx2* (Novitch et al., 2003). When RA is added to intermediate neural plate explants, then these genes and others such as *Evx1/2* and *En-1* are induced (Pierani et al., 1999). Conversely, when grown in the presence of FGF, the low levels of expression of *Pax6*, *Irx3*, *Dbx1*, and *Dbx2* in these neural explants are extinguished (Novitch et al.,

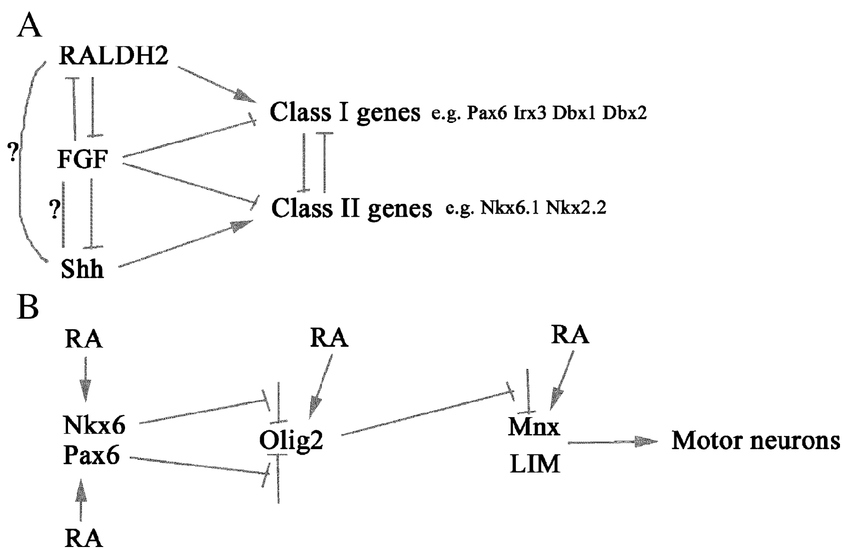

Fig. 4. Summary of the gene interactions involved in neuronal differentiation in the neural tube. (A) Network showing the relationship between the inducers of Class I and Class II genes and how they themselves interact. (B) Later neuronal differentiation of motor neurons involves multiple use of a RA signal and multiple use of the induction of repressors. See text for further details.

2003). In vivo, misexpression of a constitutively active FGFR1 or *Fgf8* itself repressed the levels of those genes and ectopic FGF prevents the onset of *Pax6* expression (Bertrand et al., 2000; Diez del Corral et al., 2002). FGF also has a limited repressive effect on two other genes not stimulated by RA, namely, *Nkx6* and *Nkx2.2* (Fig. 1C), but only when they are firstly induced by *Shh* (see below) (Novitch et al., 2003). In fact, these genes, *Nkx6* and *Nkx2.2*, tend to be repressed by RA since RA synthesis inhibition or inhibition of RA signaling allows their ectopic dorsal expansion (Novitch et al., 2003; Schafer et al., 2005). This inhibition probably operates through the RA regulation of *Pax6* rather than directly.

It is striking therefore that the inductive effects of RA on ventral genes only applies to a subset of them. Thus, *Nkx6* and *Nkx2.2* do not directly respond to RA, but they are repressed by FGF. These two genes belong to a group called Class II genes which were originally defined as being induced at particular concentrations of *Shh*; they are also unresponsive to RA. The other ventral genes, *Pax6*, *Irx3*, *Dbx1*, and *Dbx2*, are known as Class I genes which were originally defined as being repressed at distinct *shh* concentration thresholds. Now, we know that it is this class that is induced by RA and repressed by FGF (Fig. 4). Both Class I and Class II genes are homeodomain transcription factors. This brings us to consider the third molecule in this scenario, *Shh*.

Shh

Shh begins to be expressed in the notochord as soon as the cells which go to make up this structure have left the regressing node. *Shh* is, like RA and FGF, an extracellular molecule which acts in a concentration-dependent fashion. It is responsible for the embryological effects of notochord grafting (Fig. 2) as it induces the floor plate and *Shh* expression then commences within the floor plate itself (Jessell and Dodd, 1990; Placzek, 1995).

Shh, generated ventrally in the notochord and floor plate, acts in a concentration-dependent manner and is said to induce several classes of ventral interneuron progenitors (V0–V3) as well as help specify the identity of motor neuron progenitors, making 5 classes of neurons in all (Fig. 1C). This induction also occurs in vitro in a concentration-dependent manner and ectopic expression of *Shh* induces ectopic floor plate and motor neurons (Ericson et al., 1996, 1997; Marti et al., 1995; Roelink et al., 1995). However, most studies have been primarily concerned with motor neuron development and indeed the inhibition of Shh signaling with blocking antibodies stops their differentiation (Ericson et al., 1996; Marti et al., 1995). In the *Shh−/−* mutant mouse, however, V0 and V1 interneurons still develop, albeit in reduced numbers (Litingtung and Chiang, 2000), so these neuronal classes cannot be totally dependent on Shh. In fact, V0 and V1 interneurons are dependent upon RA for their development (Pierani et al., 1999; Wilson et al., 2004).

To perform its function, the *Shh* concentration gradient establishes the expression domains of the Class II genes which are activated at high levels of Shh in the ventral spinal cord (Figs. 3 and 4A). At the same time, Class I genes are induced by RA from the somites (as described above) and their ventral expression boundaries are also set by Shh because Class I genes are turned off in the ventral cord by high levels of *Shh* (Briscoe and Ericson, 2001; Jessell, 2000; Shirasaki and Pfaff, 2002). Since Class I and Class II genes encode for homeodomain transcription factors, these are the components which are the 'effectors' of the Shh gradient. Cross-repression between Class I and Class II transcription factors then establishes discrete ventral spinal cord domains that later generates specific types of neurons (Fig. 1C) (Briscoe et al., 1999; Vallstedt et al., 2001). Thus, for example, if *Pax6* is ectopically expressed in ventral regions of the chick neural tube, then *Nkx2.2* is repressed. Conversely, if *Nkx2.2* is ectopically expressed in more dorsal regions, then *Pax6* is repressed (Briscoe et al., 2000). Similarly, in the RA free quail embryo, in which Class I genes are down-regulated (see above), there is a marked dorsal expansion in the domain of the Class II gene *Nkx6.1* (Wilson et al., 2004), and in zebrafish embryos treated with an RA synthesis inhibitor, another ventral Class II gene, *Nkx2.2b*, is expanded dorsally (Schafer et al., 2005).

Despite the strength of this concentration gradient hypothesis, a gradient of Shh has never actually been detected in the spinal cord. Immunocytochemical studies only show the protein to be present in the notochord and floor plate. Nevertheless, a dorsal spread of Shh protein out of the floor plate has been inferred from two studies. Firstly, the cell surface receptor for *Shh* is patched and the *Ptc* gene is up-regulated by Shh. It is therefore a marker for Shh response, and in the neural tube, it is expressed throughout the ventral regions where Shh is acting (Marigo and Tabin, 1996). Secondly, a deleted form of *Ptc* which does not transduce the Shh signal was transfected into the chick neural tube (Briscoe et al., 2001). In cells that expressed this abnormal *Ptc*, there was a ventral to dorsal switch in gene expressions and the Class I genes *Pax6*, *Pax7*, *Dbx1*, and *Dbx2* were ectopically expressed while the Class II genes *Nkx6.1* and *Nkx2.2* were down-regulated.

Interestingly, upstream of Shh signaling from the notochord to the ventral floor plate is the Delta/Notch system (Lopez et al., 2003) and the *presenilin 1* gene (Paganelli et al., 2001) which is involved in the processing of the Notch signal. Ectopic expression of *Ps1* causes an expansion of the neural plate which is normalized by co-treatment with RA (Paganelli et al., 2001). Downstream of the Shh are the *Gli* genes which encode zinc finger transcription factors and act in the nucleus to respond to Shh signals (Ruiz i Altaba, 1999). In vertebrates, there are 3 *Gli* genes and their functions are surprisingly diverse. *Gli1−/−* mouse mutants have no discernable phenotype

(Matise et al., 1998); *Gli2*−/− mutants lack the floor plate and V3 neurons and the motor neurons extend across the midline (Ding et al., 2004; Matise et al., 1998); and in *Gli3*−/− mutants, there is a dorsal expansion of the interneuron domains (Persson et al., 2002). In the absence of both *Gli2* and *Gli3* genes, the phenotype displays characteristics of both single mutants (Lei et al., 2004). *Gli3* is thought to act as a repressor of Shh signaling and Shh levels seem to control the balance between Gli activator and repressor activities in responsive cells. Indeed, the absence of *Gli3* corrects the defect caused by the absence of *Shh*, as in the *Shh*−/−*Gli3*−/− double mutant mouse, motor neurons and interneurons develop normally (Litingtung and Chiang, 2000; Persson et al., 2002). However, this curious result suggests that Shh signaling is dispensable for DV patterning and there may be an alternative, Shh-independent ventral patterning system in place.

One aspect of Shh signaling that has not yet been addressed in any molecular detail is the relationship between *Shh*, RA, and FGF and exactly how they interact. As described above, there is a mutually antagonistic interaction between RA and FGF in the developing spinal cord which also occurs in telencephalic development (Marklund et al., 2004). The molecular details of this relationship have only been approached in studies on the AP organization of the nervous system (Shiotsugu et al., 2004) where the retinoic acid receptor α is a target of FGF signaling and, reciprocally, the expression of FGF receptors depends on the expression of the retinoic acid receptor α. Few studies have considered a *Shh*/RA or a *Shh*/FGF relationship. Some examples are a dorsal expansion of the domain of *Shh* expression in the RA free quail embryo (Wilson et al., 2004), and in *Xenopus* embryos, RA strongly down-regulates *Shh* expression and an RA antagonist up-regulates *Shh* expression in the midline (Franco et al., 1999), all suggesting a repressive effect of RA on *Shh* perhaps acting via the *presenelin 1* gene (Paganelli et al., 2001). When caudal chick tissue is cultured in FGF, *Shh* is down-regulated, suggesting a repressive effect of FGF on *Shh* (Diez del Corral et al., 2003). But as in the case of RA and FGF, no molecular details of these interactions are known.

Dorsal patterning and BMPs

Neuronal pattern in the dorsal half of the spinal cord requires the inductive activities of BMPs produced in the overlying ectoderm and roof plate (Fig. 3) (Lee and Jessell, 1999; Liem et al., 1995, 1997). The neuronal populations that are present in the dorsal half of the cord are divided into six groups (dl1–dl6) based on the expression of bHLH (*Math*, *Mash*, and *Ngn*) and LIM (*Lbx* and *Lmx*) homeodomain proteins (Helms and Johnson, 2003) (Fig. 1C).

The roof plate is the dorsal equivalent of the notochord, because when it is genetically ablated, the dorsal *Pax7* domain is reduced while the ventral *Pax6* domain expands, *Math1*+ and *Ngn*+ cells are missing, as are the respective neuronal population they produce (Lee et al., 2000). The roof plate expresses *Bmp4*, *Bmp5*, and *Bmp7* and these proteins induce dorsal markers such as *Pax3* and *Msx* and dorsal neuronal subtypes when cultured with intermediate neural plate (Liem et al., 1995; 1997). Conversely, grafts of notochord suppress the development of dorsal neurons. In zebrafish mutants which have varying degrees of compromised BMP signaling activity, there are corresponding changes in DV patterning such as loss of dorsal sensory neurons and expansion of interneurons (Barth et al., 1999; Nguyen et al., 2000). These mutants provide good evidence for a concentration-dependent mechanism of BMP action as is the case for Shh in the ventral neural tube. Additional concentration effects were seen when a constitutively active BMP receptor was electroporated or virally transfected into the chick neural tube at different expression levels (Timmer et al., 2002). As a result, *Pax7* was ectopically expressed more ventrally, *Pax6* was repressed at high levels and at lower levels was shifted ventrally, *Msx1* and *2* were induced, *Dbx1* and *2* were repressed and they showed that *Msx1* regulated *Dbx2*. Of the neuronal specification genes, *Cath1* was up-regulated whereas *Cash1*, *Ngn1*, and *Ngn2* were repressed. With regards to neuronal subtypes, Evx1 and En-1 interneurons were reduced, LH2A and B neurons were induced, Lim1/2 neurons were reduced, and dorsal interneurons expressing Islet1 were reduced or absent (Fig. 1C). Conversely, the deletion of both types of BMP receptor, *Bmpr1a* and *Bmpr1b* (but not individually), results in the loss of dl1, *Math-1* sensory interneurons and a reduction and dorsal shift in dl2 neurons (Wine-Lee et al., 2004). Thus, BMPs provide positional information in dorsal and intermediate regions by setting borders of expression of homeodomain target genes in a similar fashion to Shh ventrally.

Another member of the BMP family, Gdf7, is expressed in the chick and mouse roof plate (Lee et al., 1998). This molecule induces mATH1/cATH1 expression in adjacent dorsal neuroblasts which give rise to the dl1 neurons. In the *Gdf7*−/− mutant mouse, *Bmp6* and *Bmp7* are still expressed and a late population of mATH1 progenitors is reduced and D1A interneurons are absent. So, Gdf7 is likely to be downstream of the BMPs.

The study of a naturally occurring mouse mutant without a roof plate revealed that certain dorsal neuronal classes were missing and the gene that was affected encoded a LIM homeodomain protein, *Lmx1a* (Millonig et al., 2000). *Lmx1a* must be a target of BMPs as its ectopic expression induced an ectopic roof plate along with the *Gdf7*, *Bmp4*, and *Wnt1* genes and ectopic *Bmp4* or *7* induced *Lmx1a* (Chizhikov and Millen, 2004a). Interestingly, in the light of the early embryological experiment described above, *Lmx1a* induces expressing cells to withdraw from the cell cycle, the classical characteristic of roof plate and floor plate cells. A related gene, *Lmx1b*, that is only expressed in the chick embryo and not the mouse embryo and does not cause roof

plate cells to withdraw from the cell cycle nevertheless induces the same panoply of genes when ectopically expressed, namely, *Gdf7*, *Bmp4*, and *Wnt1* (Chizhikov and Millen, 2004b). The *Pax7* and *Msx1/2* domains were expanded ventrally, *Pax6* was reduced, and the number of dl1 neurons was increased 4-fold at the expense of dl2 and dl3 neurons. *Lmx1b* induces *Lmx1a*, but not vice versa, and so the suggested pathway is *Lmx1b* induces *Lmx1a*, which induces BMPs.

Another extracellular signal which is present in the roof plate are the *Wnts*, specifically *Wnt1* and *Wnt3a*. Rather than being involved in DV patterning, it is suggested that they are mitogens responsible for inducing proliferation in a concentration-dependent manner (Megason and McMahon, 2002). Nevertheless, the mouse *Wnt1−/−Wnt3a−/−* double mutant lacks dl1 and dl2 neurons and has excessive numbers of dl3 neurons even though the roof plate is still present and the expression of the *Bmps* and *Gdf7* is normal (Muroyama et al., 2002). In the Bmp receptor double mutant embryo, *Wnt1* and *Wnt3a* are down-regulated and the domains of their expression shrink (Wine-Lee et al., 2004). These data suggest the Wnts are downstream of the Bmps.

Do any of the other DV signals interact with BMPs? In the absence of RA signaling, there is a reduction in the expression domains of genes associated with establishing the dorsal neural pattern (Wilson et al., 2004). In particular, both *Bmp4* and *Bmp7* show a decrease and contraction in domain of expression. This is accompanied by similar effects in the downstream targets of *Bmps*, including *Msx2*, *Pax3*, and *Pax7*. Such results suggest that RA is involved in the regulation of dorsal patterning genes and the concomitant expansion of *Shh* and Class II genes in the RA free ventral cord would suggest that the dorsal phenotype is due to the antagonistic relationship between ventral and dorsal genes (Jessell, 2000; Liem et al., 1997, 2000; Wilson et al., 2004). However, dorsal expression of the RA-catabolizing enzyme *Cyp26A1*, accompanied by the presence of *Raldh2* in the roof plate at later stages (Berggren et al., 1999; Blentic et al., 2003; Swindell et al., 1999), may indicate the existence of local RA-mediated patterning mechanisms within the dorsal spinal cord and this remains to be tested. Indeed, RA-deprivation causes abnormal roof plate and floor plate formation in the spinal cord, as well as a reduction in neural tube size and cell number (Wilson et al., 2003).

The next phase: specification of neuronal subtypes

The networks of transcription factors that have been set up by FGF, RA, Shh, and BMP signaling, as described above, not only act by directly inducing subsequent target genes, but also direct cell fate through the repression of other repressors (Barolo and Posakony, 2002; Lee and Pfaff, 2001; Mannervik et al., 1999; Muhr et al., 2001). In this way, the emergence of an individual cell type is achieved by the repression of alternative cell fates, in a de-repressive manner (Muhr et al., 2001; Thaler et al., 2004). This is particularly true of the motor neurons (MNs), where most of the transcription factors involved in specification function as repressors (Lee and Pfaff, 2001; Shirasaki and Pfaff, 2002). For example, the expression of *Olig2* marks MN progenitor state and the co-expression of *Nkx6* and *Pax6* is required to prevent the expression of transcription factors capable of repressing *Olig2* expression (Briscoe et al., 2000; Lu et al., 2002; Novitch et al., 2001; Sander et al., 2000). *Olig2* itself then functions as a transcriptional repressor to direct the expression of downstream homeodomain regulators of MN identity via Mnx (Mnr2 and Hb9) and LIM (Isl1/2 and Lim3) proteins (Mizuguchi et al., 2001; Novitch et al., 2001; Rowitch et al., 2002; Scardigli et al., 2001; William et al., 2003), indicating a de-repression mechanism is functioning during this later phase of MN specification also (Fig. 4B).

RA is required again in this second phase for progression to MN specification. In vitro explants and RA free embryos were used to show that RA signaling is required for *Nkx6* positive cells to progress to an *Olig2* positive state in MN progenitors (Diez del Corral et al., 2003; Novitch et al., 2003). Co-electroporation of a dominant-negative RA receptor and *Olig2* showed that RA provides the transcriptional signal necessary (either in parallel or downstream of *Olig2*) for the expression of MN-specific genes as well as promote pan-neuronal differentiation (Novitch et al., 2003), a finding which is reflected in the down-regulation of MN differentiation genes *Hb9*, *Mnr2*, and *Isl-1* in the absence of RA (Wilson et al., 2004). Interestingly, joint exposure of neural progenitors to retinoids and FGFs (that normally inhibit Class I genes) is sufficient to induce MN differentiation in a Shh-independent manner (Novitch et al., 2003).

Thus, the retinoid-mediated transcriptional activation of neuronal subtype specification occurs in three sequential steps in MNs: firstly, RA-bound receptors activate the expression of *Pax6* (a Class I gene), and thus provide a de-repressed context in which *Olig2* expression is permitted; secondly, RA activates the expression of *Olig2* in de-repressed ventral progenitors; and thirdly, RA acts downstream of *Olig2* expression to activate the expression of homeodomain transcription factors that confer MN identity, and bHLH proteins that promote pan-neuronal differentiation (Fig. 4B).

Rostrocaudal (RC) and DV interactions in the neural tube

The neuronal subtypes present in the cord vary along the RC axis and where a neural progenitor cell exits the caudal stem zone along the RC axis determines its DV fate and character. So how does a neuroblast undergoing DV patterning know where it is on the RC axis? The RC axis of the vertebrate spinal cord can be broadly divided into five

domains based on regional distinctions reflected in the position and projection of specific cell types, namely, the cervical, brachial, thoracic, lumbar, and sacral domains. Many of the neuronal classes found in the DV spinal cord are generated along the entire RC axis, but motor neurons (MNs) exhibit marked RC differences in identities that have been defined through studies of their position, axon trajectory, and pattern of muscle innervation (Landmesser, 2001). For example, limb muscle-innervating MNs form a discontinuous lateral motor column (LMC) only at brachial (forelimb) and lumbar (hindlimb) levels whereas body wall-innervating MNs form a median motor column (MMC) along the whole length of the spinal cord. At thoracic levels, sympathetic neuron-innervating MNs form the column of Terni (CT) neurons in chick (Gutman et al., 1993; Hollyday, 1980a,b). A further division exists as LMC neurons are subdivided into medial and lateral subtypes comprised of MNs that innervate ventral or dorsal limb muscles (Hollyday and Hamburger, 1977; Landmesser, 1978a,b).

Major distinctions in the RC identity of spinal MNs have been attributed to actions of certain members of the *Hox* gene family as their expression and functional profiles correlate with the RC positional identity of MNs (Belting et al., 1998; Bel-Vialar et al., 2002; Carpenter, 2002; Ensini et al., 1998; Lance-Jones et al., 2001; Liu et al., 2001). Grafting studies in chick have provided evidence for the upstream regulation of *Hox* genes and show that the positional identity of spinal MNs, as well as the pattern of *Hox* gene expression, can be respecified soon after neural tube closure by signals derived from the paraxial mesoderm (Ensini et al., 1998; Muhr et al., 1999).

What are the signals from the paraxial mesoderm—the same signals that are acting on DV patterning, presumably at the same time, namely, RA and FGF. Caudal mesoderm expresses higher levels of FGF8 than does rostral mesoderm and low levels of FGF induce the expression of rostral Hox proteins such as Hoxc5 or Hoxb4, whereas high levels of FGF induce the expression of the caudal proteins such as Hoxc9 or Hoxb9 (Bel-Vialar et al., 2002; Liu et al., 2001). RA is well known as a *Hox* gene regulator (Simeone et al., 1990) and there may be subtle differences in the expression levels of RALDH2 in the paraxial mesenchyme along the RC axis with higher levels anteriorly (Berggren et al., 1999). Cervical paraxial mesoderm plus retinol (the precursor of RA) induces the rostral Hoxc5 and inhibits the posterior proteins, Hoxc8 and Hoxc9 (Liu et al., 2001). This induction is inhibited in the presence of RA receptor antagonists which inhibit RA signaling. Therefore, the idea has developed that the RC regions of the neural tube are defined by the following combinatorial paraxial mesoderm signals: cervical, high RA; brachial, low RA, low FGF; thoracic, high FGF; lumbar, high FGF, high Gdf11. These signals establish general Hoxc domains which then undergo cross-repression with each other, creating sharp definitive boundaries at different RC levels in the cord (Dasen et al., 2003; Harris, 2003; Liu et al., 2001). Thus, the boundary between Hoxc6 and Hoxc9 establishes the boundary between the LMC of the cervical cord and the CT of the thoracic cord.

The concept that RA is responsible for defining the cervical and brachial regions of the neural tube is supported by several studies including those on the alterations in DV patterning in the RA free quail embryo which have been referred to above. The changes in DV gene domains only occur in the rostral (cervical and brachial) regions of the spinal cord (Wilson et al., 2004). A rostral-specific loss of spinal cord ventral interneuron gene expression is also observed in the zebrafish *neckless* mutation (a mutation in the *Raldh2* gene) (Begemann et al., 2001). In addition, a role for paraxial RA specifically defining MN type in rostral regions is provided by experiments in which levels of RA signaling were manipulated by electroporating MNs with dominant-negative or constitutively active RA receptor constructs (Sockanathan et al., 2003). Inhibition of retinoid receptor signaling in brachial, but not lumbar MNs prevented the acquisition of LMC identity as assessed by gene expression profile, neuronal settling profile, and axonal projection patterns. Instead, these neurons became thoracic level CT and lateral MMC neurons despite their brachial position.

Therefore, FGF8 and RA released from the presomitic mesoderm not only control onset of differentiation and ventral neural pattern formation in the extending body axis, but also regulate the pattern of Hox proteins, which in turn specifies motor column identity. This illustrates how the acquisition of DV identity of cells within the spinal cord greatly depends on when and where the cell is born in respect to the RC axis, and that these events employ the repeated use of convergent signaling systems.

The final phase: specification of motor neuronal subtype

Once high RA/low FGF from the paraxial mesoderm and Shh from the notochord and floor plate has generated firstly, MN identity and then brachial LMC identity, further RA signaling is required to distinguish between the two subtypes of LMC neurons, lateral LMCs (LMCL) and medial LMCs (LMCM). The former innervate dorsal limb muscles and the latter innervate ventral limb muscles and are located in discrete pockets in the ventral horns (Fig. 5A).

Up to now, RA has been synthesized outside the neural tube in the paraxial mesoderm, but from stage 19 in the chick and day 12.5 in the mouse, Raldh2 begins to be expressed in the MNs themselves at brachial and lumbar levels, but 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) and this endogenous synthesis of RA is clearly the reason why. LMCL neurons are born later than

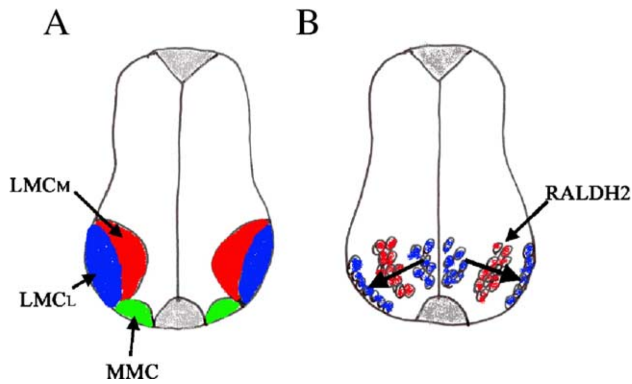

Fig. 5. Differentiation of subsets of motor neurons. (A) Drawing to show the location of the medial lateral motor column neurons (LMCM, red), the lateral lateral motor column neurons (LMCL, blue), and the medial motor column neurons (MMC, green) in the mature spinal cord. (B) The LMCL neurons (blue) are born after the other LMCs and have to migrate from their site of origin at the ventricular surface to their settling position at the lateral edge of the cord (black arrow). In doing so, they pass through RALDH2 expressing LMCs (red) which generate RA and induce the LMCL phenotype in those migrating neuroblasts.

other LMC neurons and they are located at the lateral extremity of the ventral horn. Since neurons are born towards the ventricular surface, presumptive LMCLs have to migrate through earlier-born LMCs (Fig. 5B). It is the earlier-born LMCs that express *Raldh2* and synthesize RA which induces the LMCL phenotype in the late-born neuroblasts as they migrate through. RA increases the number of Isl+ neurons (a general marker of MNs) in cultured thoracic (non-limb level) and neural tube cultured in the presence of RA and cultured brachial (limb level) cultured have decreased numbers of LMCLs when in the presence of RA receptor antagonists which inhibit RA signaling (Sockanathan and Jessell, 1998). When thoracic (non-limb) neural tube was transfected with *Raldh2*, many LMCLs were induced, but they were not the cells which had been transfected; instead, they were adjacent to the transfected neuroblasts. This suggests that LMCLs are induced in a non-autonomous fashion.

Summary and speculations

We have described what might be called the 4-signal model for DV patterning in the neural tube. FGF is expressed in the caudal mesoderm and must be down-regulated before either Class I or Class II neural genes can be induced. RA is produced by the enzyme RALDH2 in the paraxial mesoderm and presumably diffuses into the neural tube where it induces Class I genes and diffuses caudally into the mesoderm where it represses FGF. Shh is produced by the notochord and floor plate and in a concentration-dependent manner induces ventral Class II genes. BMPs are produced by the roof plate and in a concentration-dependent manner induces dorsal genes and dorsal neuronal types. Class I and II genes are transcription factors, and in order to establish stable domains of expression (Fig. 1C), there are cross-repressive interactions between these transcription factors. RA is utilized several times more in the sequence of events leading to motor neuron differentiation to induce transcription factors which repress repressors and thus allow differentiation. Finally, RALDH2 is expressed by a subset of early-born motor neurons, and during the migration of later-born motor neurons through this RALDH2 domain, the RA so generated induces LMCLs to differentiate.

This patterning system (Fig. 3) is an excellent example of how morphogenetic signals are controlled in development. A signal, once it has done its job, is then switched off by the next signal which then does its job and so on. But only some of these interactions have been identified and there are several which must remain the subject of further research. Thus, FGF represses RA and RA represses FGF. RA seems to repress Shh, because in the absence of RA, the Shh domain expands (Wilson et al., 2004), but the relationship between Shh and RA has not been investigated. Similarly, FGF seems to repress Shh (Diez del Corral et al., 2003), but whether Shh represses FGF is not known. Nor has the relationship between RA and the dorsal genes been investigated apart from the observation that in the absence of RA, the BMPs and Wnts are down-regulated (Wilson et al., 2004). There is therefore much to learn about these interacting networks of morphogenetic molecules.

Two other aspects of this patterning process have not really been addressed. One is the role of Wnts in DV patterning. As mentioned earlier, Wnts have been ascribed a role as positive regulators of proliferation rather a role in patterning, yet the removal of *Wnt1* and *Wnt3a* results in the disappearance of specific dorsal neuronal types rather than a general decrease in proliferation (Muroyama et al., 2002). This raises the long-standing debate in development of the relationship between patterning and proliferation and which drives which. Another curiosity is the *Shh*/*Gli3* double knockout which has normal ventral patterning, suggesting that Shh is dispensable for ventral patterning despite the strong basis on which the Shh story has been built.

It is interesting to consider whether these same interactions between morphogenetically active molecules occur in other regions of the embryo. In the limb, for example, RA, FGFs, BMPs, and Shh all play a part in patterning. The antagonistic relationship between RA and FGF does seem to exist here (Mercarder et al., 2000), but with regard to RA and Shh, RA induces Shh in the limb (Riddle et al., 1993), which does not seem to occur in DV patterning because RA seems to repress Shh (Wilson et al., 2004). So it seems that in different developing systems, the relationship between interacting gene networks is different.

Finally, it is clear from the above description of the processes of DV patterning that there is already a complex web of interactions in the DV plane of the neural tube, but added to that complexity is the fact that at the same time as DV patterning is occurring, RC patterning is also occurring. This involves both RA and FGF, again in an antagonistic

relationship. One obvious question which arises from all this complexity is how does a neural cell interpret all these conflicting messages? How does a cell distinguish between, for example, an RA signal which is intended for RC information and an RA signal which is intended for DV information? There is clearly still much to learn about this remarkable embryonic process.

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