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Patterning of the vertebrate ventral spinal cord

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ABSTRACT We review investigations that have lead to a model of how the ventral spinal cord of higher vertebrate embryos is patterned during development. Central to this model is the secreted morphogen protein, Sonic hedgehog. There is now considerable evidence that this molecule acts in a concentration-dependent manner to direct the development of the spinal cord. Recent studies have suggested that two classes of homeodomain proteins are induced by threshold concentrations of Sonic hedgehog. Reciprocal inhibition between the two classes acts to convert the continuous gradient of Sonic hedgehog into defined domains of transcription factor expression. However, a number of aspects of ventral spinal cord patterning remain to be elucidated. Some issues currently under investigation involve temporal aspects of Shh-signalling, the role of other signals in ventral patterning and the characterisation of ventral interneurons. In this review, we discuss the current state of knowledge of these issues and present some preliminary studies aimed at furthering understanding of these processes in spinal cord patterning.

KEY WORDS: *spinal cord, patterning, Sonic hedgehog, interneuron, motor neuron*

Introduction

The vertebrate central nervous system (CNS) is an incredibly complex organ system that performs many tasks including physiological regulation, collection and processing of sensory information, control of movement and locomotion, and the formation of memories and learning. In humans, and perhaps some other mammals, the CNS is also responsible for higher functions such as language and consciousness. Understanding how this organ system is constructed during embryonic development would provide a fascinating insight into these processes as well as greater understanding of CNS disease. It is no surprise that many research laboratories are currently focused on this problem. This article reviews some aspects of this work. Development, in its true sense, spans the lifetime of the organism, however we have confined discussion to early and middle stages of embryonic development, and focussed on studies in two animal models, mouse and chick.

There are a number of approaches being taken to uncover the biology of the developing CNS. Two of the most popular involve the study of developing axon projections and synaptic connections, and a focus on unravelling epigenetic cascades. The latter approach stems from the premise that specific cell fates result from molecular restrictions and involves the identification of genes essential for CNS development.

Many genes required for normal development are highly conserved and encode transcription factors, cell surface adhesion molecules, receptors or signalling molecules. These genes show restricted spatial and temporal patterns of expression throughout the course of neural development. By studying both the mechanisms that dictate their expression and the putative roles of these genes during CNS development, models of epigenetic cascades that give rise to adult CNS structure and function can be constructed.

To date, studies of molecular mechanisms required for the development of the CNS have focused on the developing spinal cord. This portion of the CNS has important roles in sensory perception, reflex generation and movement and locomotion. The spinal cord is also singled out for more pragmatic reasons as it is structurally and functionally simpler than the brain and it is hoped that the molecular mechanisms operating in the spinal cord can be extended to the higher centres.

Abbreviations used in this paper: A-P, anterio-posterior; BMP, bone morphogenetic protein; CNS, central nervous system; d*pc*, days *post coitum*; D-V, dorsoventral; HD, homeodomain; Shh-N, N-terminal portion of the Sonic hedgehog protein.

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† This article is dedicated to Toshiya Yamada (15th October 1960 – 12th May 2001)

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Morphogenesis of the Vertebrate Spinal Cord

The vertebrate CNS develops from the ectoderm germ layer. Neural induction specifies the neural plate in which subsequent involution gives rise to a cylindrical structure termed the neural tube (Schoenwolf, 1982). In mammals and birds, the neural tube initially consists of a single layer of multipotent cells, referred to as the ventricular zone. Waves of proliferation from this multipotent layer give rise to both neurons and glia. These cells undergo specific programmes of differentiation and migration before acquiring their final functional form (Fig. 1).

As the number of cells increases, the spinal cord increases in girth and length. It acquires two more layers referred to as the mantle (or intermediate) and marginal zones. The cell bodies of post-mitotic, differentiating cells are confined to the mantle zone, giving rise to a distinct grey appearance. Within this zone, some neurons develop local connections amongst themselves and others send forth axons to distal targets and, in the process, form the marginal layer (Rakic, 1982). Glial cells gradually encapsulate the axons of the marginal zone to give this zone its distinctive white appearance in the adult spinal cord. The grey matter gradually becomes butterfly shaped due to both the invaginating lateral *sulcus limitans* at the division between the dorsal and ventral halves, and the settling of cells in the dorsal and ventral horns.

Regionalisation of the Developing Spinal Cord

The developing spinal cord progressively becomes regionalised along the dorso-ventral (D-V) and anterio-posterior (A-P) axes. Regionalisation along the A-P axis results in organisation of neurons according to their different neuraxial targets. Patterning of the A-P axis is conferred by a number of molecular signals from the paraxial mesoderm such as retinoids and members of the fibroblast growth factor (FGF) and Wnt protein families (Ensini *et al.*, 1998; Lumsden and Krumlauf, 1996), as well as the combinatorial expression of *Lim* homeobox genes and *Hox* genes (Jungbluth *et al.*, 1999).

Before describing the D-V organisation of neurons, it is important to note that along the dorsal and ventral midlines are the respective roof plate and floor plate. These are non-neuronal structures that have cardinal roles in molecular aspects of neuronal development and patterning. Neurons of the mantle layer are classified along the D-V axis into two dorsal (alar) columns and two ventral (basal) columns flanking the roof and floor plates respectively.

The alar columns flanking either side of the roof plate give rise to several neuron groups including some with sensory function. The basal columns separated by the floor plate give rise to somatic motor neurons and ventral neurons with various projections within the CNS (Brown, 1981) and now loosely termed ventral interneurons. These broad neuronal domains can be further subdivided to contain neurons of similar morphology, trajectory and physiology. Since neuronal groups are defined by their D-V positions, the factors that pattern the D-V axis are important for the specification of neuronal identities.

Dorso-Ventral Patterning is Controlled by Signals Emitted from Organising Centres

Embryonic manipulations in chick and genetic approaches in rodents have confirmed that the D-V polarity of the developing spinal cord is specified by the action of extracellular signals that emanate from organising centres (Briscoe *et al.*, 1999, 2000; Ericson *et al.*, 1997; Goulding *et al.*, 1993; Lee *et al.*, 2000; Liem *et al.*, 1995, 1997; Yamada *et al.*, 1991; 1993). Signals that specify the dorsal neural tube are secreted by the ectodermal endoderm and later by the roof plate, and ventralising signals emanate from the notochord and later from the floor plate.

The patterning of dorsal fates is mediated through coordinate actions of several TGF-β family proteins, notably BMP -4 and -7 (Liem *et al.*, 1995), while ventral patterning of the neural tube is mediated by the Sonic hedgehog (Shh) protein (Roelink *et al.*, 1995). Both Shh and the BMPs are involved in activation or repression of transcription factors, leading to the specification of progenitor domains and the expression of genes that define distinct neuronal fates or cell-types. This review will now focus on patterning of the ventral spinal cord and a recent model of this

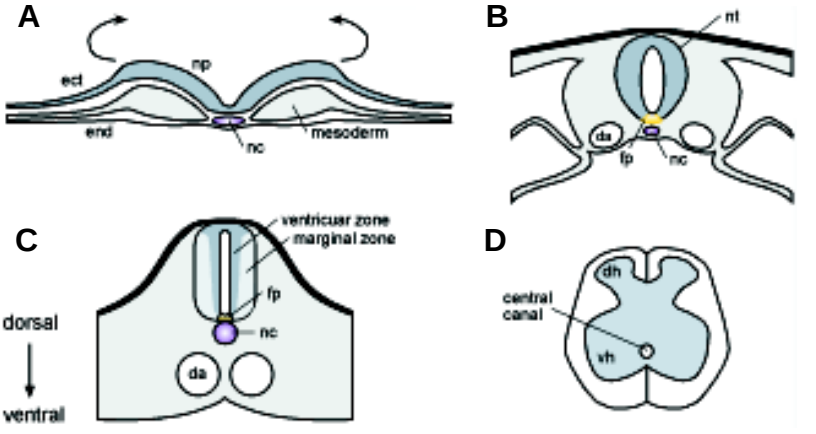

Fig. 1. **Diagrammatic representation of the developing ventral neural tube. (A)** *The neural plate (np) is derived from the ectoderm germ layer (ect) and is the source of all neuronal and glial cells. The neural tube is formed by the neural plate rolling up along its rostral-caudal axis (arrows) and a fusion of the lateral extents of neural plate. Signals from the notochord (nc) are active at this early stage.* **(B)** *The early neural tube (nt) consists entirely of mitotic precursor cells. Shh-signalling from the notochord acts as an initial ventralising influence and also induces the non-neuronal floor plate tissue (fp).* **(C)** *Shh-signalling from both the notochord and floor plate continue to pattern the precursors of the ventral spinal cord. More ventrally located populations start specific differentiation programmes and exit the cell cycle earlier than more dorsal populations. These cells migrate laterally to form the marginal zone. Some undifferentiated progenitors remain in the ventricular zone and continue to divide.* **(D)** *The mature spinal cord consists of the cell bodies of differentiated motor neurons and interneurons (the grey matter) which have migrated to form the ventral and dorsal horns (vh, dh). The surrounding white matter consists of axons and glial cells. These diagrams are based on transverse sections of the developing chicken but are generally applicable to mammals. da, dorsal aorta; end, endoderm.*

process (Briscoe *et al.*, 2000; Muhr *et al.*, 2001). In addition, we raise some current issues under investigation such as temporal aspects of Shh-signalling, the role of other signals in ventral patterning and recent insights into spinal interneuron development.

Shh-N is Responsible for Ventral Neural Tube Patterning

Numerous studies have confirmed that the notochord, a cylindrical structure that forms ventral to the neural tube, is the initial source of ventralising signals (reviewed by Jessell 2000; Jessell and Melton, 1992). In neural explant studies, notochord tissue induced the expression of ventral molecular markers (Yamada *et al.*, 1991, 1993). The notochord is the source of two operationally distinct inductive signals; a short-range signal which acts locally to induce floor plate differentiation along the midline of the neural tube, and a long-range signal that patterns motor neuron and ventral interneuron differentiation (Ericson *et al.*, 1996).

It was found that the notochord was a source of the vertebrate hedgehog protein (Yamada *et al.*, 1993), an important morphogen first characterised in *Drosophila* (Lee *et al.*, 1992). Evidence strongly suggested that Sonic hedgehog, as it was termed, was the diffusible factor responsible for the inductive activities of the notochord (Roelink *et al.*, 1994). Floor plate cells induced by notochord signalling also acquired the ability to synthesise Shh. Furthermore, Shh is synthesised when both the notochord and floor plate exhibit their ventral patterning activities and mis-expression of Shh was able to induce an ectopic floor plate *in vivo* (Chiang *et al.*, 1996; Goodrich *et al.*, 1996). Studies with neural explants found that recombinant Shh induced the differentiation of floor plate and motor neurons, and the addition of antibodies inhibiting Shh signalling counteracted this effect (Martí *et al.*, 1995; Roelink *et al.*, 1995). It has been shown that autoproteolytic cleavage of Shh generates an N-terminal fragment (Shh-N) that is responsible for the biological activity of the molecule (Martí *et al.*, 1995; Porter *et al.*, 1995, Roelink *et al.*, 1995). Cholesterol is covalently attached to the C-teminus of this protein fragment to anchor the molecule in the plasma membrane, and there is evidence that the N-terminus is palmitoylated (Pepinsky *et al.*, 1998; Porter *et al.*, 1996a, b). In addition to these lipid modifications, there are other indications that lipids play important roles in Shh signalling (reviewed by Ingham, 2001).

A critical role for Shh-N in the development of ventral structures in the CNS was formally demonstrated in mice carrying a targeted mutation of the *Shh* gene (Chiang *et al.*, 1996). *Shh*$^{-/-}$ mouse embryos demonstrated severe holoprosencephaly (HPE); a condition characterised by development of the prosencephalic derivatives as a single undivided vesicle consisting of fused eye fields, remnants of the dorsal telencephalic lobes, and an absence of ventral forebrain structures such as the optic stalks, the optic chiasm, and the pituitary (Belloni *et al.*, 1996; Chiang *et al.*, 1996). Externally severe HPE is characterised by an absence of midline facial structures and the development of a proboscis consisting of fused nasal chambers at a location overlying a cyclopic eye (Cooper *et al.*, 1998). Loss of function mutations at the human *Shh* locus are associated with a milder and more variable form of HPE that is inherited in an autosomal dominant fashion, indicative of haploinsufficiency (Belloni *et al.*, 1996).

A Concentration Gradient of Shh-N patterns the Ventral Spinal Cord

Investigation of gene expression in the developing spinal cord has revealed a number of genes with expression domains restricted along the D-V axis. Comparison of these expression domains with anatomical knowledge of the spinal cord suggested that these genes might define groups of neuronal progenitors in the ventricular zone or differentiating, post-mitotic neurons in the mantle zone. For example, all post-mitotic motor neurons could be identified by the combined expression domains of the Lim-homeodomain genes *Islet1* and *Islet2* (Ericson *et al.*, 1992; Tsuchida *et al.*, 1994). Similarly, expression of several *Pax* genes in the ventricular zone were used to define potential progenitor domains, and homeobox genes such as *Chx10*, *En1* and *Evx* were used to demarcate post-mitotic and differentiating interneuron populations (Burrill *et al.*, 1997; Ericson *et al.*, 1997; Liem *et al.*, 1997; Matise and Joyner, 1997; Pfaff *et al.*, 1996). These genes encode transcription factor proteins, suggesting that they might play a role in the genetic cascades that specify distinct neuron cell types.

Investigations designed to manipulate the notochord and floor plate in chick embryos or by utilising an *in vitro* chick explant assay system, demonstrated that the expression of these molecular markers could be influenced by Shh-N protein. During formation of the neural tube, the dorso-ventrally restricted expression of a number of homeodomain (HD) genes in the ventricular zone of the neural tube was changed in response to altered Shh-N exposure (Goulding *et al.*, 1993; Ericson *et al.*, 1996, 1997; Liem *et al.*, 1995). Importantly, Shh-N protein appeared to exert this influence in a concentration-dependent manner (Ericson *et al.*, 1996, 1997). A close correlation was found between changes to the expression domains of these molecular markers and the subsequent differentiation of cell types in the spinal cord.

The D-V expression of molecular markers in post-mitotic cells was also influenced by Shh-N. As previously mentioned, the expression domain of these genes was used to demarcate populations of post-mitotic motor neurons and ventral interneurons. It was discovered that the expression of these genes and thereby the differentiation of distinct neuronal classes along the D-V axis was controlled by threshold concentrations of Shh-N protein (Ericson *et al.*, 1997). Good evidence for a morphogenic gradient of Shh-N was provided by examining the distribution of the Shh-N protein (Ericson *et al.*, 1996; Marigo and Tabin, 1996) and later by mutation of components of the Shh-N receptor complex (Briscoe *et al.*, 2001; Hynes *et al.*, 2000).

These and other studies have led to a model where signalling by a concentration gradient of ventral Shh-N controls the expression of HD patterning genes in the mitotic progenitor cells of the ventricular layer. These genes, in turn, activate different downstream identity genes in dorso-ventrally-restricted populations of post-mitotic neurons. The pattern of certain ventral neuronal subtypes is perturbed in mice carrying mutations in *Nkx2.2* and certain *Pax* genes (Briscoe *et al.*, 1999; Ericson *et al.*, 1996, 1997; Mansouri and Gruss, 1998), supporting the view that HD proteins expressed by ventral progenitor cells regulate neuronal subtype identity.

Recently, this model has been refined by the identification of more HD transcription factor genes expressed in dorso-ventrally restricted populations of neuronal progenitors under the control of

secreted Shh-N (Briscoe *et al.*, 2000). This refined model, based on previous work and *in vivo* and *in vitro* functional assays performed by Briscoe *et al.* (2000), proposed that ventral neural tube patterning occurs in three major stages (Fig. 2). In the first stage, a gradient of Shh-N signalling activity establishes the D-V domains by influencing expression of HD transcription factors of two categories: Shh-N repressed (the so-called class I proteins including Pax6, Pax7, Dbx2 and Irx3) and Shh-N activated (referred to as class II proteins including Nkx6.1 and Nkx2.2, Fig. 2A). This results in five ventral progenitor domains. Reciprocal repression between class I and class II proteins then acts to refine and maintain these progenitor domains. Thirdly, the expression profile of Shh-N repressed proteins and Shh-N activated proteins determines future neuronal fate: distinct classes of post-mitotic motor neurons or interneurons that can be visualised by dorsoventrally restricted gene expression. The beauty of this model is that the repressive interactions between the Shh-N-defined domains of the class I and II proteins translates the continuous Shh-N concentration gradient into defined domains of progenitor cells. More recent studies have identified two more transcription factors that could be included in the class II group (Mizuguchi *et al.*, 2001; Novitch *et al.*, 2001; Vallstedt *et al.*, 2001). These are Nkx6.2 and Olig2, although both these factors differ from the other class II members in that the ventral extent of their expression is restricted (Fig. 2). Further, Olig2 is not a HD protein; rather it is a member of the basic helix-loop-helix family.

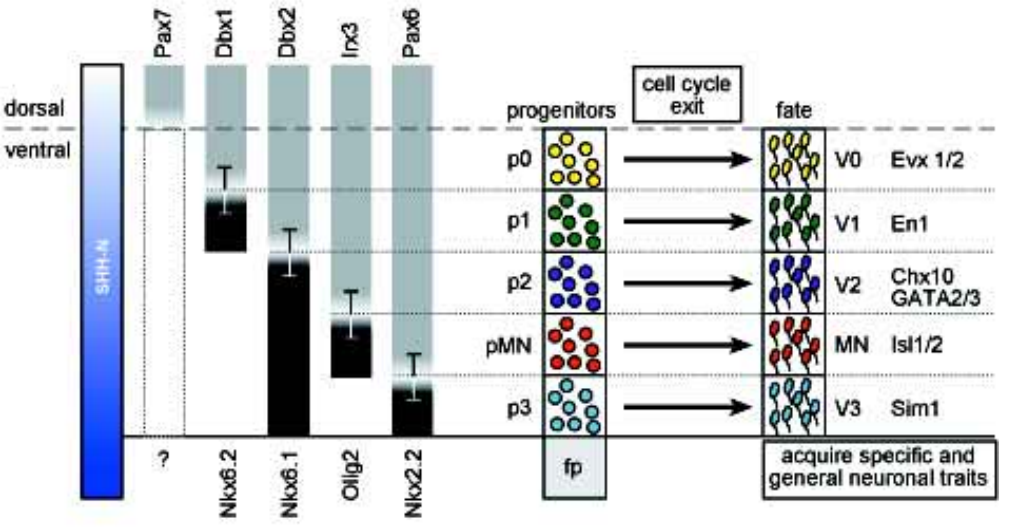

Fig. 2. The ventral spinal cord is patterned by translation of a Sonic hedgehog gradient into domains of transcription factor expression. *In the current model of ventral patterning a continuous concentration gradient of Sonic hedgehog protein (Shh-N) acts at specific threshold concentrations to repress or induce expression of transcription factors within progenitor cells. These factors are termed class I (grey) and class II (black) respectively. Reciprocal repression between class I and class II factors that have adjacent extents of expression acts to refine the boundaries between expression domains. In this way, a continuous morphogen gradient is translated into distinct populations of progenitor populations (p0-3 and pMN) demarcated by transcription factor expression. The combinatorial expression of transcription factors then acts to drive progenitors out of the cell cycle and into specific differentiation programmes (motor neuron, MN, and ventral interneuron fates V0-3). Post-mitotic cells then acquire general and specific neuronal traits. The latter can be recognised by the specific expression of other transcription factors. The possible presence of an, as yet, uncharacterised class II factor is shown. The division between the dorsal and ventral halves of the spinal cord is shown as a dashed line. fp, floor plate.*

Temporal Aspects of Ventral Patterning by Shh-N Signalling

On analysis of anatomy and ventral marker expression it is evident that the development of some neuronal populations precedes that of others. This suggests the establishment of particular neural fates at independent time points. However, the inductive signal (*i.e.* Shh) responsible for the generation of many ventral neural subtypes present very early in CNS development and remains for some time after populations of neurons have embarked upon a particular differentiation pathway. For example, precursor cells are exposed to Shh-N while the CNS is at the neural plate stage (Fig. 1A), however the first neurons to differentiate (motor neurons) appear some 18 hours later (Ericson *et al.*, 1992; Liem *et al.*, 1995, Pfaff *et al.*, 1996). Further, motor neurons and other classes of interneurons continue to appear still later (Langman *et al.*, 1966; 1970). An obvious question is; how are different cell-populations established at different times by an inductive signal that has a continuous presence?

This question has been investigated in a number of studies focussing on when precursors require a signal of a specific concentration and how long they require, or can respond to, this signal (Fig. 3). One such study by Ericson *et al.* (1996) used chick neural explant assays and recombinant Shh protein or Shh-N blocking antibodies to examine the timing of motor neuron development. They found evidence for two distinct stages.

Firstly, precursor populations that respond to Shh must be 'primed' by a low level of Shh-N protein at an early stage in development. This priming signal acts to 'ventralise' a portion of the neural tube and this ventralisation can be visualised by the repression of Pax7 expression. Apparently, Shh-N is required for at least some time to maintain the ventralised state, as tissue removed from Shh-N exposure at stage 10 (staging criteria of Hamburger and Hamilton, 1951) reverted to a Pax7-positive state. In contrast, tissue removed at approximately 12 hours later at stage 12 did not re-express Pax7, thereby suggesting a period of required signalling after which the cells become independent. This low signal was not enough to induce specific cell types. To produce motor neurons, Ericson *et al.* (1996) needed to expose ventralised tissue to a second stage of higher (ten-fold) concentration of Shh-N. Explanted tissue cultured in the absence of Shh-N for 12 hours was not able to produce motor neurons even when subsequently exposed to this higher concentration, suggesting a period of competence after which the naïve cells become unresponsive to Shh-N signal.

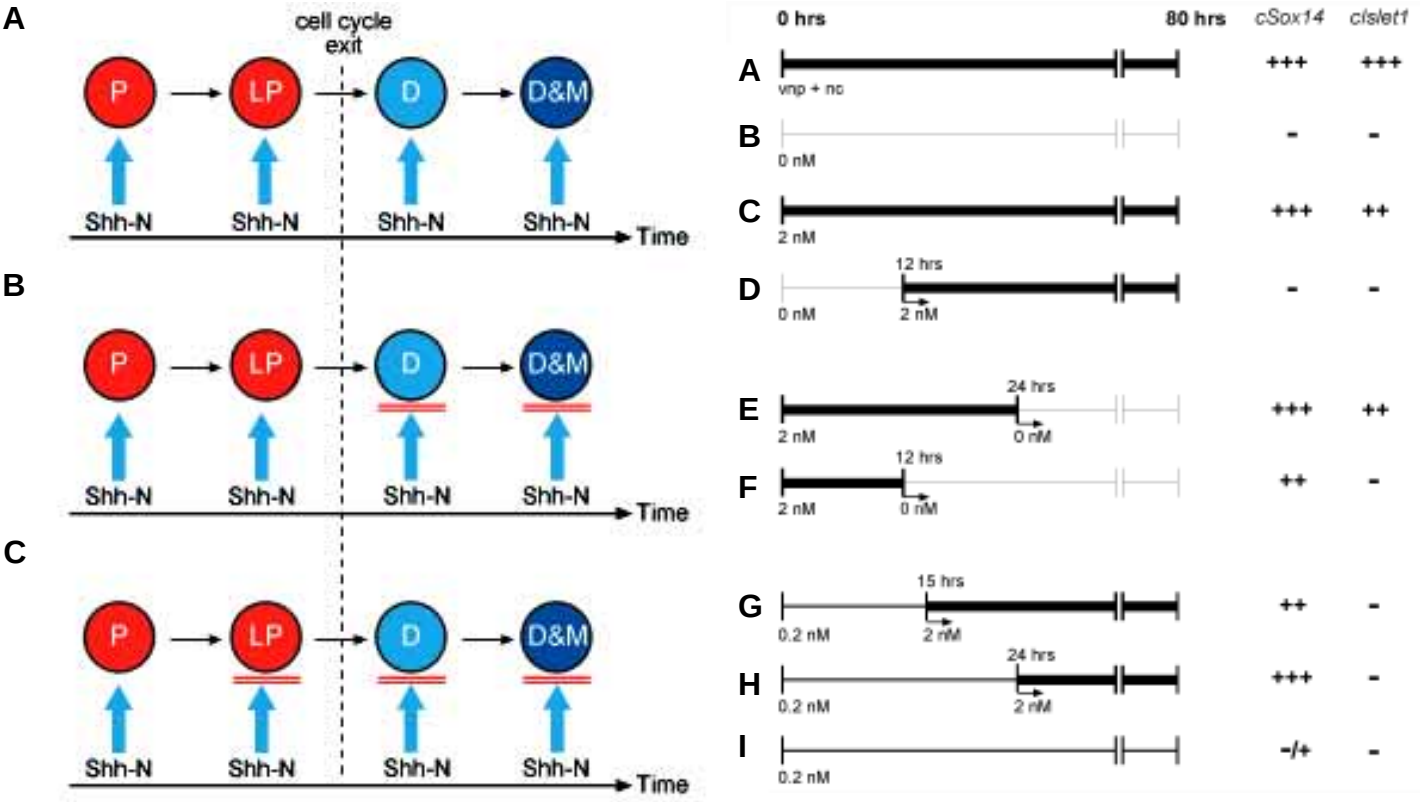

Fig. 3. (Left) How long do cells in the ventral spinal cord respond to Shh-N? *The extended period of Shh-N availability during ventral patterning raises the question of how long cells require or can respond to the signal.* **(A)** *A model of ventral spinal cord patterning where neurons require or are able to receive Shh-N signalling for an extended period. The first stage represented is an early multipotent progenitor state (P). This is followed by a stage where an instructive signal has been received and the cell has committed to a developmental programme (late progenitor, LP). The cells then exit from the cell cycle (dashed line) and express the first molecular markers of differentiation (D). Cells then undergo further differentiation and migration (D&M).* **(B)** *A model where cells become refractory or independent of Shh-N-signalling after cell cycle exit and/or commencement of differentiation.* **(C)** *A model supported by the investigation of Ericson* et al. *(1996) which observed that motor neuron progenitors become independent of Shh-N in their final S phase.*

Fig. 4. (Right) Competence and independence of progenitors to Shh-N. *Diagrammatic representation of the response of ventral neural plate (vnp) explant tissue to recombinant Shh-N protein administered for varying time periods.* **(A)** *Explants cultured for 80 hours in the presence of notochord tissue (nc) are able to generate* Islet1$^+$ *motor neurons and* Sox14$^+$ *V2 interneurons.* **(B)** *Neither cell type can be generated by naïve explants.* **(C)** *Both cell types can be induced by exposure to 2 nM recombinant Shh-N protein although* **(D)** *this exposure must occur before 12 hours in culture.* **(E,F)** *To produce motor neurons, the explants require exposure to Shh-N for a critical time between 12 and 24 hours in culture. In contrast, V2 interneurons become independent of Shh-N less than 12 hours after exposure.* **(G,H)** *Despite early exposure to the 'priming' or ventralising concentration (0.2 nM) described by Ericson* et al. *(1996), explants require exposure to the higher 'inducing' concentration of Shh-N (2 nM) before 15 hours in culture to be able to generate motor neurons. Progenitor cells in the explants remain competent to produce V2 interneurons at least 24 hours after ventralisation (although the initial signal is required, compare with D).* **(I)** *The ventralising signal alone is not sufficient to produce either cell type.*

Ericson *et al.* (1996) then tested the requirement of continued high level signalling for the development of motor neurons by using a Shh-N blocking antibody and bromodeoxyuridine (BrdU) to label dividing cells. They observed that motor neuron precursor cells become independent in their final S phase shortly before the appearance of post-mitotic motor neurons. It is tempting to speculate that this independence might be correlated with the formation of sharp boundaries between progenitor domains by the reciprocal inhibitions of HD proteins (see Fig. 2).

We also sought to investigate temporal aspects of cell-type specification within the chick spinal cord and also performed neural plate explant culture assays, adding different concentrations of recombinant Shh proteins at different timeframes (Fig. 4). We then examined changes in expression of two ventral marker genes, *Sox14* and *Islet1*. This allowed us to monitor the differentiation of both motor neurons, marked by *Islet1*, and a class of ventral interneurons, marked by *Sox14* (Hargrave *et al.*, 2000). Like Ericson *et al.* (1996), we observed a requirement for a low concentration 'priming' signal of Shh-N to subsequently induce cell fate, and that this signal had to be received within a critical period of less than 12 hours after initiation of explant culture. Similarly, this priming signal alone was not able to induce specific cell types. Again, a 10-fold concentration of Shh-N was required.

We also found that after a critical period, Shh-N was no longer required for the differentiation of cell types. In other experiments using cyclopamine (see below) to inhibit Shh-N signal transduction, we found further evidence for the development of Shh-N-independence in a variety of developing ventral cell types (A. Poh and T. Yamada, unpublished observations). However, we observed that this critical period was different for the motor neurons

and *Sox14*-positive interneurons. The time window at which motor neurons become Shh-N- independent *in vitro* lies between the 12th and the 24th hour (Fig. 4). In contrast, the *Sox14*-positive ventral interneurons appear to gain independence at a very early stage, as they do not appear to be significantly affected by the removal of Shh-N after 12 hours.

We then tested the competence of primed precursors to give rise to either of the two cell fates (Fig. 4). Again we found a difference between the two populations. In response to a higher Shh-N concentration, precursors that give rise to *Sox14*-positive interneurons can remain competent to the appropriate Shh-N for a considerable length of time (>24 hours *in vitro*), where those which give rise to *Islet1*-positive motor neurons lose their ability to respond earlier in development (<15 hours *invitro*).

From these preliminary studies it appears that for fates that require a high dose of Shh-N, the inductive signal has to be provided to primed precursors very early (short competence) and maintained for an extended period of time for Shh-N- independence to be attained. In contrast, the more dorsal low-dose fates can receive the signal 'reasonably late' (longer competence) and gain independence earlier. There is some evidence that naïve neural plate or ventralised (primed) precursors without subsequent Shh-N adopt an interneuron-like fate (as judged by expression of Lim1/2, Ericson *et al.*, 1996). Perhaps the difference in temporal aspects of motor neuron and *Sox14*-positive interneurons is due to the greater 'distance' of the former from this 'default state'. It is not clear if the timing mechanisms governing ventral cell-type specification are intrinsic or otherwise (*i.e.* perhaps 'priming' is similar to a 'timer' mechanism, where once the precursors are 'primed' the 'timer' is switched on).

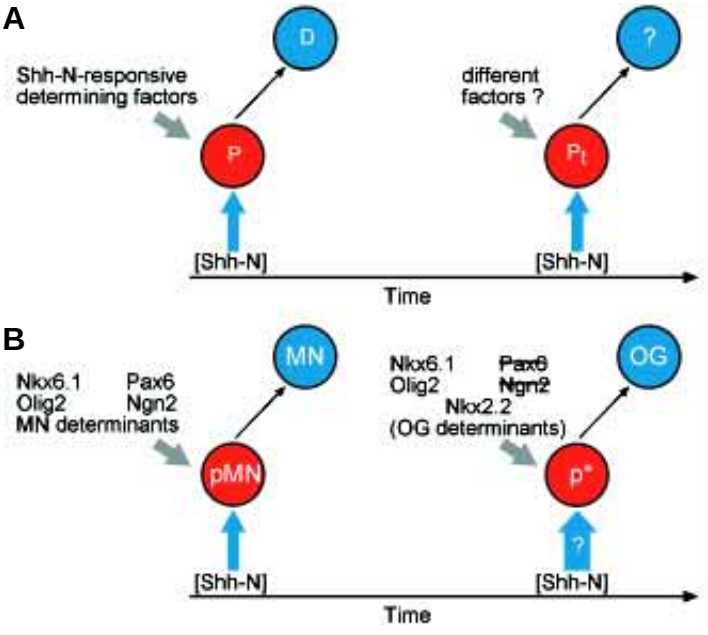

Fig. 5. Changing response of progenitors to Shh-N signalling over time. (A) *The presence of the Shh-N protein for an extended period in ventral spinal cord development raises the possibility that precursors (P) at a defined dorso-ventral position may change their response over time (P_t) to the Shh-N concentration at that position. This change in response may arise from an induction of different Shh-N responsive transcription factors (grey arrows).* **(B)** *The region of the spinal cord (pMN) that initially gives rise to motor neurons (MN) later produces oligodendrocytes (OG). Progenitors in this region receive a Shh-N signal that induces expression of factors that drive motor neuron differentiation. After a defined period, progenitors (p*) express Nkx2.2 but not Pax6 or Ngn2 causing oligodendrocytes to be produced. It has been suggested that the change in transcription factor expression is due to a local increase in Shh-N concentration rather than an intrinsic timing mechanism within progenitors that alters their response to a given Shh-N concentration. See text for references.*

Using a complementary strategy of Shh-N over-expression in mice, Rowitch *et al.*, (1999) found more evidence for timing mechanisms in spinal cord development. By creating a transgenic mouse strain that ectopically expressed Shh-N in the spinal cord from 10 d*pc* (days *post coitum*) they observed that while the ectopic Shh-N was able to direct ectopic expression of ventral markers in the dorsal spinal cord, it was unable to induce a floor plate fate. This suggested that the ectopic Shh-N expression occurred after a critical period of competence for floor plate induction. Further, Rowitch *et al.* (1999) observed that the ectopic Shh-N was able to promote proliferation within the spinal cord at 12.5 d*pc* but not at 18.5 d*pc*, prompting them to suggest the existence of a 'clock' that restricts the period of precursor proliferation.

Another question is how progenitor cells respond to a continued, perhaps varying, exposure to Shh-N (Fig. 5). For example, the present model predicts that ventralised progenitors that receive an appropriate concentration of Shh-N will give rise to motor neurons. Observation of motor neuron development has revealed that they appear for a defined period (Ericson *et al.*, 1992; Liem *et al.*, 1995, Pfaff *et al.*, 1996). After this period, oligodendrocytes are produced in a Shh-N-dependent manner at the same dorso-ventral position that was previously the birthplace of motor neurons (see Richardson *et al.*, 2000; Soula *et al.*, 2001; Zhou *et al.*, 2001 and references therein). The two distinct cell types, neuronal and non-neuronal, could be produced as a result of one progenitor population switching its developmental programme. Alternatively, two juxtapositioned but distinct progenitor populations may each give rise to one type. In either scenario, some mechanism to input temporal information is required.

Evidence suggested that the former model of a single precursor switching its output was the more likely (reviewed by Richardson *et al.*, 2000). Recent investigations have revealed a major molecular player in this process, the basic helix-loop-helix (bHLH) transcription factor Olig2 (reviewed by Marquardt and Pfaff, 2001). During motor neuron specification, Olig2 expression is induced by the class I and II HD proteins (Pax6 and Nkx6.1) that define the motor neuron progenitor domain, pMN (Figs. 2 and 5). Olig2 then indirectly induces motor neuron fate through de-repression of motor neuron determinants (Mizuguchi *et al.*, 2001; Novitch *et al.*, 2001; Sun *et al.*, 2001). Olig2 also appears to promote cell cycle exit and the acquisition of general neuronal characteristics through the de-repression of another bHLH protein, Neurogenin2 (Ngn2). This last observation opens up exciting possibilities to investigate long-standing questions regarding these processes.

Later, a dorsal expansion of the class II HD factor Nkx2.2 overlaps with expression of Olig2 to define a new progenitor domain, p* (Fig. 5; Zhou *et al.*, 2001). The combination of these two factors appears to promote oligodendrocyte specification over that of motor neurons by an, as yet, unclear process that probably includes repression of Ngn2 (Marquardt and Pfaff, 2001; Soula *et al.*, 2001; Zhou *et al.*, 2001). An increase in Shh concentration has been suggested as the cause for the expansion of Nkx2.2 expres-

sion, although how, and why, this might occur is not clear. If there is an increase in Shh-N secretion from the floor plate, then this could have interesting consequences for the other progenitors in the ventral spinal cord.

Transduction of the Shh Signal

The Shh signal transduction pathway is highly complex and its involvement in development and tumourigenesis has made it the subject of intense investigation (reviewed in Kalderon, 2000; Wicking and McGlinn, 2001). Since various components of the intracellular Shh signalling cascade are conserved through evolution much of what is known about this highly complex cascade has been drawn from studies in *Drosophila*. The receptor for Shh-N is a 12-pass transmembrane protein Patched (Ptc, Marigo *et al.*, 1996). In the absence of Shh-N, Ptc inhibits the 7-pass transmembrane protein, Smoothened (Smo) which is responsible for the transduction of the hedgehog signal. In the presence of Shh-N, this Ptc-mediated inhibition of Smo is alleviated and the transduction pathway is initiated. The Shh signal is then transduced to nuclear effectors, the Gli zinc-finger transcription factors, which are regulated via a number of cytoplasmic molecules. It is possible that different levels of Gli activity may repress or activate the class I and class II homeobox genes proposed by Briscoe *et al.* (2000).

How Ptc regulates Smo activity is still under debate, although it appears that the cellular location of the Ptc and Smo proteins is an important issue. Recent studies in *Drosophila*, indicate that Ptc and Smo are largely localised within the cell (Martin *et al.*, 2001; Strutt *et al.*, 2001). This has lead to the hypothesis that Ptc may inhibit Smo by an indirect mechanism rather than as a direct physical interaction of the two molecules at the cell surface (reviewed by Kalderon, 2000).

A naturally occurring inhibitor of the Shh-N signal transduction pathway has been identified in a lily plant, *Veratrum californicum* (Cooper *et al.*, 1998; Incardona *et al.*, 1998). This steroidal alkaloid molecule, aptly termed cyclopamine (11-deoxojervine), caused cyclopia in lambs when ingested by pregnant ewes (Binns *et al.*, 1963; Keeler, 1969). The mechanism of inhibition by cyclopamine is still not established, though it is thought to exert an effect on Smo (Taipale *et al.*, 2000). Investigation of a number of Shh-N dependent cell types both *in vivo* and *in vitro* has demonstrated that essentially all aspects of Shh-N-mediated dorso-ventral patterning are interrupted upon exposure to cyclopamine (Cooper *et al.*, 1998). Ventral cell types normally induced by Shh-N are absent, or appear ectopically along the ventral midline whereas the domains of the dorsal Shh-N-repressed cell types extend ventrally.

While using cyclopamine to investigate temporal aspects of Shh-N-mediated ventral patterning, we observed an interesting difference in the behaviour of motor neuron markers Isl1/2 and SC1. As previously discussed, all post-mitotic motor neurons are identified by Isl1/2 transcription factor expression (Ericson *et al.*, 1992; Tsuchida *et al.*, 1994). Post-mitotic motor neurons can also be identified by the expression of the cell-surface adhesion protein SC1 (Tanaka *et al.*, 1991). Cyclopamine treatment at three different stages of chick development (stages 10, 15 and 20) resulted in the predicted reduction of the Shh-N dependent Isl1/2. Similarly the SC1 was almost entirely abolished in stage 10 treated embryos. However, cyclopamine treatment at stages 15

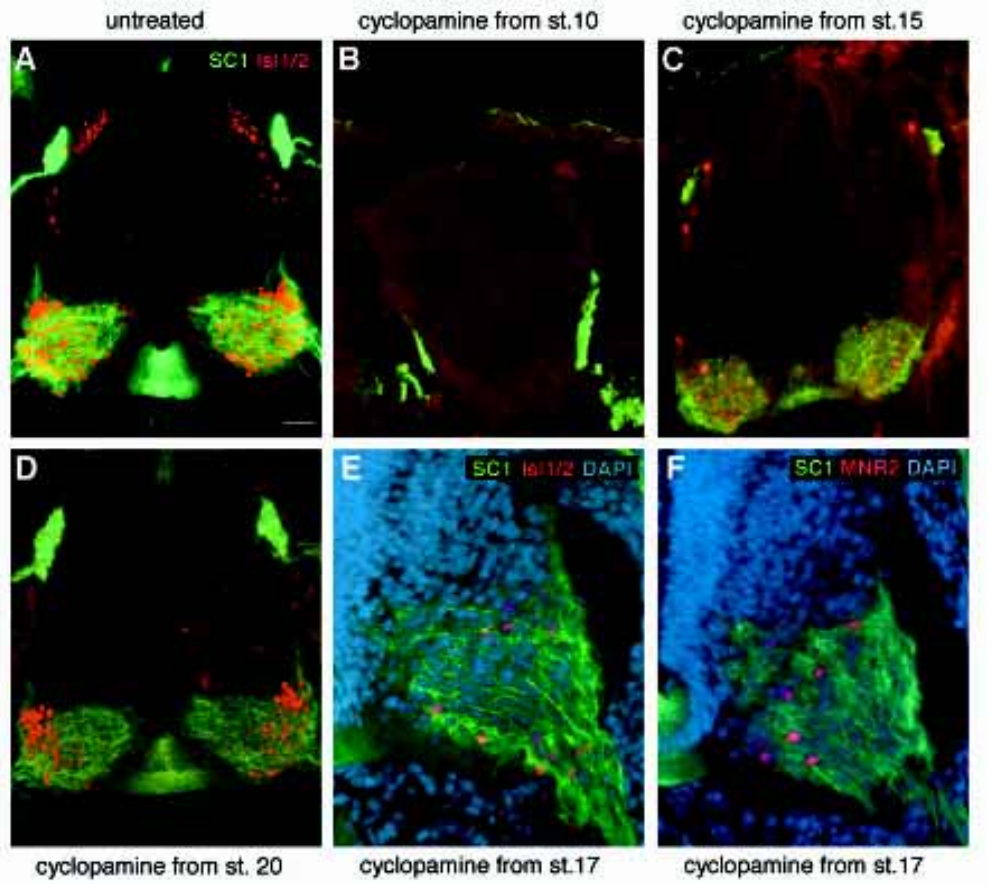

Fig. 6. Motor neuron markers respond differently to cyclopamine treatment. *(A-E) Double immunofluoresence labelling of lumbar-sacral sections of the developing chick spinal cord with two types of Shh-dependent motor neuron markers, SC1 (green) and Isl1/2 (red).* **(A)** *A representative section of an untreated embryo showing expression of the cell surface protein SC1 and nuclear transcription factors Isl1/2 in motor neurons. SC1 is also expressed in the floor plate and dorsal root entry zone and Isl1/2 in a population of dorsal interneurons.* **(B)** *Inhibition of Shh-N signal transduction by cyclopamine from stage 10 onwards severely disrupts ventral patterning. Both SC1 and Isl1/2 are absent.* **(C)** *Expression of SC1 is largely unaffected in Stage 15 and* **(D)** *Stage 20 treated embryos despite the reduction of Isl1/2.* **(E)** *Labelling of stage 17 treated embryos with the nuclear marker DAPI indicates that SC1 protein is associated with viable cells.* **(F)** *A similar observation was made using another motor neuron marker, MNR2. All panels are representative sections of multiple experiments. Scale bar, 100 μm.*

and 20 did not result in a major reduction of SC1 expression, despite the large reductions in Isl1/2 expression (Fig. 6). SC1 expression in the neural tube has been demonstrated to be dependent on Shh-N in several investigations. These include ectopic floor plate and notochord grafting experiments in chick embryos (Ericson *et al.*, 1992; Placzek *et al.*, 1991; Yamada *et al.*, 1991), ectopic *Shh* expression by electroporation in chick embryos (Watanabe *et al.*, 2000) and in neural explant studies (Yamada *et al.*, 1993).

Presumably the abolition of SC1 expression in stage 10 treated embryos is due to a failure of ventralisation or 'priming' of precursors (see discussion above and Ericson *et al.*, 1996). Why there is a strong difference in cyclopamine-induced effects between motor neuron markers after this point is not clear. It is possible that there is a mechanistic difference in the signal transduction or subsequent induction of transcription factors that leads to expression of each marker. Recently, evidence for cyclopamine-insensitivity has been shown in neural crest cell migration, another Shh-N-dependent process. In this system, Shh-N mediates the migration of neural crest cells by the suppression of the cell adhesion molecule integrin (Testaz *et al.*, 2001). Shh-N-mediated migration of neural crest cells was not suppressed by cyclopamine, nor was it inhibited by other compounds known to block signal transduction, including forskolin, cyclohexamide and endomycinD. As these compounds target specific aspects of signal transduction, it was proposed that the effect of Shh-N on neural crest cell migration was not mediated by the Ptc/Smo/Gli signalling pathway.

Another possibility is that SC1 expression is influenced by other signals. Mice with targeted mutations that apparently abolish Gli2/3-mediated Shh-N-signalling still have certain ventral interneurons and some motor neurons (Litingtung and Chiang, 2000), suggesting that some ventral fates are induced by other signals (reviewed by Stone and Rosenthal, 2000). There is mounting evidence for such signals (see section below) and their effects on ventral spinal cord patterning. In any case, the identity of cells that express one so-called motor neuron marker but not another is an intriguing question.

The Contribution of Other Signals to Ventral Patterning

While there is no doubt that the Shh protein plays a major role in the patterning of the ventral spinal cord, there is evidence for the involvement of other signalling molecules. Differentiating motor neurons migrate laterally from the ventricular zone and are subdivided into a number of columns. The medial motor column innervates the axial and body wall muscles and the lateral motor column, located at limb levels only, innervates the dorsal and ventral limb muscles (see Sockanathan *et al.*, 1998 and references therein). An early born population of lateral motor neurons were found to secrete retinoids which signalled to a later born population of cells to migrate more laterally and form the lateral segment of the lateral motor column (Sockanathan *et al.*, 1998). In another investigation, it was also shown that while Shh-N was sufficient to induce VO and V1 interneurons, it was not necessary. Retinoid signalling appeared to be able to induce these cell types independently of Shh-N (Pierani *et al.*, 1999).

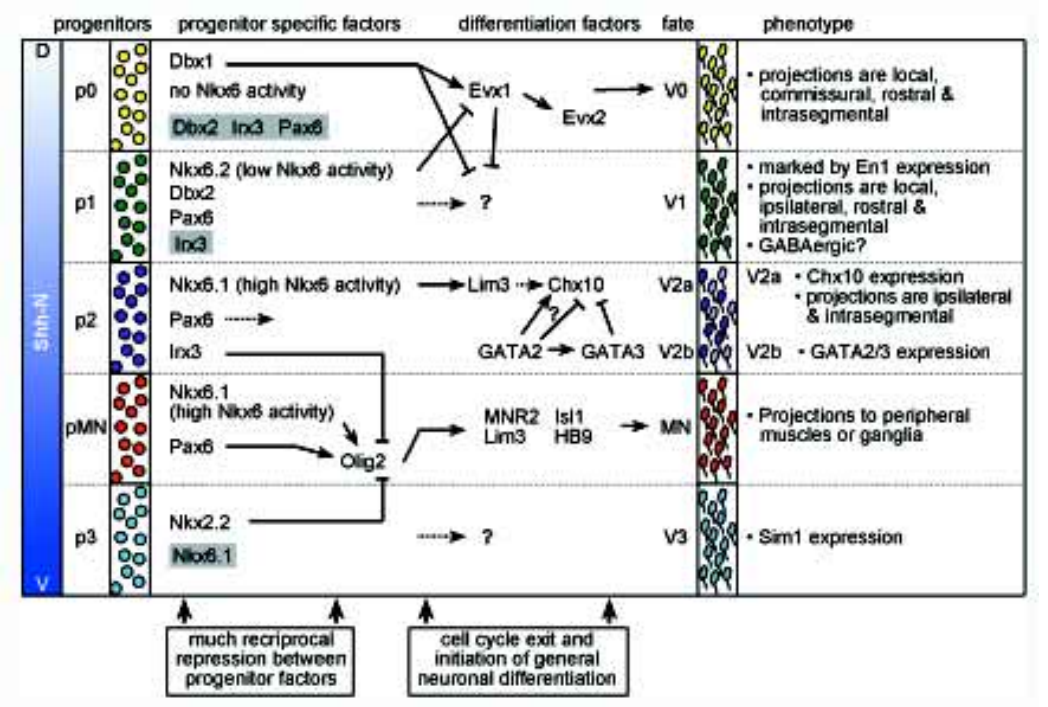

Fig. 7. Summary of the molecular events that give rise to ventral cell fates. *The many instances of reciprocal repression between class I and class II factors that are expressed in progenitors in response to Shh-N-signalling are not shown. Molecular events with supporting evidence are shown as solid lines while those that are inferred or assumed are shown as dotted lines. Factors that are expressed in progenitor populations but with unknown functions in those progenitors are enclosed in grey boxes. The factors that drive differentiation of the V1 and V3 interneurons have not been identified. Chx10 and GATA2/3 are included with the differentiation factors, however their role in this process is not proven. The uncertainty of the effect of GATA2 expression on that of Chx10 is shown (see text and Fig. 8). The processes that cause progenitors to exit the cell cycle and acquire general neuronal characteristics are largely unknown. See text for references.*

As previously mentioned, BMPs, members of the TGF-β superfamily, have been shown to have critical roles in dorsal-ventral patterning (Lee *et al.*, 1998, 2000; Liem *et al.*, 1995, 1997). While much of the focus on BMPs has concentrated on their potent role in dorsal patterning there also appears to be a role for BMPs in patterning of ventral cell types. This is supported by several reports of BMP expression in and around the ventral neural tube at various stages during vertebrate neural development (Barth *el al.*, 1999; Bruneau *et al.*, 1997; Jones *et al.*, 1991; Liem *et al.*, 1995; 2000; Nguyen *et al.*, 2000; Streit *et al.*, 1998). In addition, there is evidence from mutation and over-

expression studies of BMP inhibitors. Mice homozygous for a null allele of *Noggin*, which encodes a BMP2/4 inhibitor, displayed severe ventral patterning defects, including the loss of a fully functional floor plate and a reduction of motor neurons and ventral interneurons in the posterior spinal cord (McMahon *et al.*, 1998). Furthermore, over-expression of follistatin, an inhibitor of BMP7, leads to expansion of ventral cell types (Liem *et al.*, 2000). This suggests that BMPs as well as Shh-N are required for ventral cell type specification and that their availability needs to be precisely controlled.

We have been investigating the role of a potential BMP binding protein termed Crim1 (Kolle *et al.*, 2000). Crim1 is a putative transmembrane-bound or secreted protein with multiple cysteine rich repeats, similar to those found in the BMP4 extracellular antagonist, chordin (Piccolo *et al.*, 1996). Intriguingly, *Crim1* is expressed in both mouse and chick in the floor plate and subsets of the motor neuron pool after neural tube closure. Due to the recent findings implicating BMP signalling in the development of the ventral neural tube, we suggest that Crim1 may modulate the function of one or numerous BMPs in ventral cell type specification and subsequent differentiation.

Molecular Aspects of Interneuron Development

Motor neurons with their conspicuous soma and early differentiation in the ventral spinal cord were often the major focus of investigations of spinal cord development. The many molecular studies since have made a major contribution to the description of motor neuron development (reviewed by Goulding 1998; Jessell, 2000). In contrast, embryonic interneurons in both avian and rodent species have not been well characterised, and the description of these cells was largely restricted to their projection patterns (Oppenheim *et al.*, 1988; Silos Santiago and Snider; 1992, 1994). Unlike motor neurons, which have relatively simple axon projections, adult interneurons of different classes project axons to many sites within the spinal cord and brain (Brown, 1981). The current challenge is to connect differential gene expression in interneurons with the development of distinct axon projection patterns.

Like motor neurons, it appears that combinatorial expression of transcription factors directs the development of functional classes of interneurons. Four distinct classes of ventral interneurons have been proposed based on the combinatorial patterns of transcription factor gene expression (Figs 2 and 7; Briscoe *et al.*, 2000; Burrill *et al.*, 1997; Ericson *et al.*, 1997; Matise and Joyner, 1997). All four classes of ventral interneurons occupy distinct dorso-ventral positions in the developing spinal cord and are regulated by the graded Shh signal. Over the past five years, a number of investigations have revealed molecules involved in the specification of these classes and, in some cases, a description of axonal projections and neurotransmitter profiles (summarised in Fig. 7; Briscoe *et al.*, 2000; Moran-Rivard *et al.*, 2001; Pierani *et al.*, 1999, 2001; Sander *et al.*, 2000; Saueressig *et al.*, 1999; Vallstedt *et al.*, 2001; Wenner *et al.*, 1999, 2000). Of particular interest was a recent study of the role of the closely related Nkx6.1 and Nkx6.2 proteins in interneuron development. By utilising single and double knockout strategies in mice, this investigation suggested that a delicate balance of cross-repression between transcription factor proteins is required for the generation of multiple cell fates (Vallstedt *et al.*, 2001).

Also of interest was the observation that the Engrailed-1 (En1) protein was not required for the establishment of the post-mitotic V1 cell type despite the fact that En1 expression is used by neurobiologists to define the V1 class (Saueressig *et al.*, 1999). En1 is apparently only required for some later aspects of the post-mitotic differentiation of the V1 interneuron population. This might caution us that distinctions based on gene expression alone might not truly reflect underlying anatomy. Still, the presumption that all En1 expressing cells share something in common was by no means radical and this type of approach has provided testable models. Reassuringly, a similar study found that the 'marker' for V0 interneurons, Evx1, is required for correct differentiation of the majority of V0 interneurons (Moran-Rivard *et al.*, 2001). A lack of Evx1 caused many V0 neurons to acquire features of V1 neurons and over-expression of Evx1 repressed that of En1.

Recent investigations in our laboratory have revealed that at least one of these groups of developing interneurons, the V2 group, can be subdivided at an early stage (A. Karunaratne, M.Hargrave

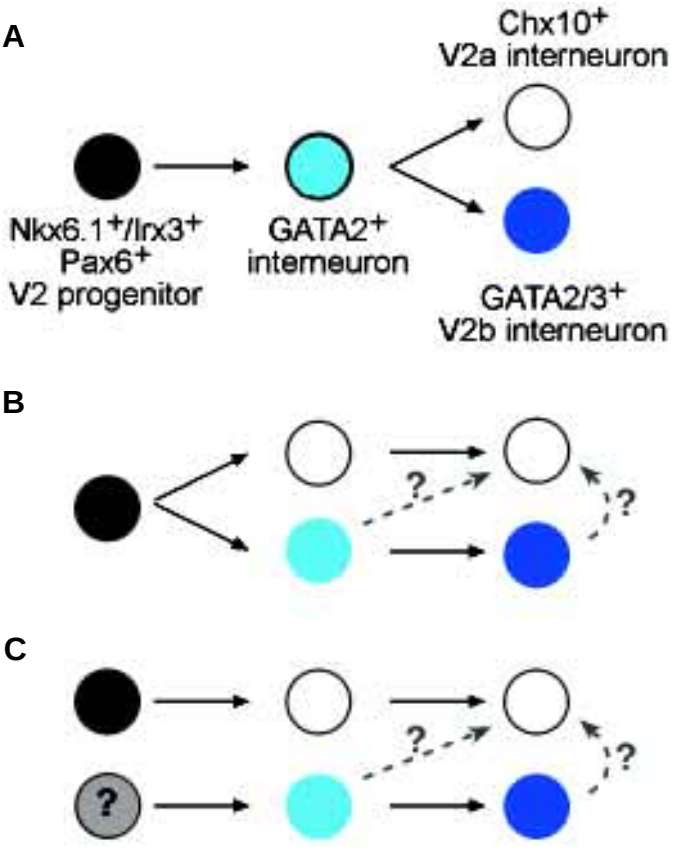

Fig. 8. Proposed models for V2a and V2b interneuron generation. (A) *Generation of V2a and V2b interneurons by a population of early differentiating cells that express GATA2. This model requires the cessation of GATA2 expression in cells that go on to express Chx10 (V2a interneurons). This model is supported by the observed lack of Chx10+ interneurons in the GATA2 knockout mouse. In contradiction to this model, we observed that GATA2 and Chx10 appear simultaneously in the chick spinal cord.* **(B)** *A single progenitor population independently gives rise to both Chx10+ V2a interneurons and GATA2/3+ V2b interneurons. The local presence of the V2b interneurons is required for the survival of V2a interneurons (dashed arrows).* **(C)** *V2a and V2b interneurons are generated from two independent progenitor domains. This model evokes the existence of two distinct progenitor populations at the same dorso-ventral position in the spinal cord.*

and T.Yamada, unpublished observations). The V2 population is demarcated by the homeodomain protein Chx10 and, in mice, requires the GATA2 zinc-finger transcription factor protein for its development (Ericson *et al.*, 1997; Zhou *et al.*, 2000). When we compared the expression of Chx10 with that of GATA2 and the related GATA3 in chick we found that Chx10 and GATA2/3 are expressed by distinct populations of interneurons that share the same dorso-ventral position. No co-expression was observed between the GATA proteins and Chx10, even when the two populations were induced by *in vivo* notochord grafting or by recombinant Shh-N in neural tube explants. The two types of cells were induced together but as intermixed cells expressing either factor. Further evidence suggested that GATA2 precedes and induces expression of GATA3 and that the two GATA proteins are capable of repressing Chx10 expression. We designated the Chx10-positive cells as V2a interneurons and the GATA2/GATA3-positive neurons as V2b interneurons (Fig. 7). Although located within the same motor pool, different subclasses of motor neurons that innervate specific peripheral targets can be identified by the combinatorial expression on Lim homeodomain factors (Tsuchida *et al.*, 1994). Similarly, while intermixed within the same dorso-ventral location, V2a and V2b interneuron populations are likely to carry different functional properties, as specified by their transcription factor gene expression.

As previously mentioned, a loss of GATA2 activity in mice leads to a significant reduction in Chx10 expression (Zhou *et al.*, 2000). The same study also suggested that loss of GATA2 had no effect on the differentiation of other ventral cell types. Perhaps Chx10-postive cells are generated from a GATA2-expressing lineage where expression of GATA2 is down regulated prior to that of Chx10 (Fig. 8A). GATA2 may therefore identify a population of multipotent cells that give rise to two different ventral interneuron subtypes. This proposal is not supported by our observations that GATA2 and Chx10 appear in the developing spinal cord at the same time. Alternatively, the V2 progenitor subtype (Nkx6.1- and Irx3-positive) might generate both V2a and V2b populations. The specific loss of Chx10 expression in the spinal cord of *GATA2* knockout mice might be explained by a requirement of a 'local presence' of V2b neurons for V2a neuron survival (Fig. 8B). A third possibility is that these two populations are derived from two independent progenitor populations (Fig. 8C). However, this would also require a mechanism that could generate alternative precursor populations at the same time and dorso-ventral position.

Towards an Understanding of How Animals Build Circuits

It would appear that the reductionist approach reviewed here has provided some insight into the mechanisms of how cells in the spinal cord acquire their diverse anatomical and physiological characteristics. While, there is still some way to go, it is now conceivable that we will start to link observations of developmental processes with those of physiologists who have probed the nature of neurons in the juvenile or adult spinal cord. Especially exciting is the prospect that studies of the type summarised here and those investigating axon guidance will help us understand how circuits are built. Sensory perception, proprioception, reflexes and rhythm generation are only some of the tasks performed by simple and complex spinal circuits. Unravelling these circuits will help us understand locomotion, co-ordination and posture. Finally, it is hoped that these studies will somehow help us to understand the more complex issue of circuit formation in the brain.

Materials and Methods

Fertilised white leghorn chicken eggs (Ingham Enterprise, Brisbane) were incubated in a humidified, forced-draft incubator until they reached appropriate stages of development (Hamburger and Hamilton, 1951). Stage 10 chick intermediate neural plate explant assays was performed according to a previously published method (Yamada *et al.*, 1993). Explants were cultured in the presence or absence of notochord tissue or defined concentrations of purified recombinant, N-terminal Shh protein (corresponding to amino acid residues between 1-198, batch 5066, a gift of Drs Thomas Jessell and Susan Morton, Columbia University, NY). Expression of *Islet1* and *Sox14* was detected by RT-PCR using a previously published protocol (Hargrave *et al.*, 2000) *In ovo* cyclopamine treatment of chick embryos was performed according to the published methods with a modification (Incardona *et al.*, 1998). Cyclopamine (kindly provided by Dr. William Gaffield, USDA USA) was dissolved at a concentration of 1 mg/mL in 10 mM PBS with 10 mg/mL of HBC. 10 μL of the stock solution was injected into stage 10, 15 or 20 chick embryos with daily repeated injection up to stages 24-25. The cyclopamine-treated embryos were fixed and examined by immunofluoresence as previously described (Yamada *et al.*, 1991). The mouse anti-Isl1/2 (4D5 and 2D6) and anti-MNR2 (81.5C10) monoclonal antibodies were developed by Thomas M. Jessell and Susan Morton. They were obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD and maintained by The University of Iowa, Department of Biological Sciences (Iowa City, Iowa USA). The rabbit anti-SC1 polyclonal antibody was kindly provided by Dr Hideaki Tanaka (Kumamoto University, Kumamoto, Japan). The primary antibodies were detected using a Cy3-conjugated goat anti-mouse IgG antibody (Jackson ImmunoResearch Laboratories, Inc., West Grove, Pennsylvania, USA) or a BODIPY-conjugated goat anti-rabbit IgG antibody (Molecular Probes, Inc., Eugene, Oregon, USA). The antibody labelling was observed on an Olympus AX70 compound fluorescent microscope. Immunofluorescence images were obtained using an MTI digital camera and NIH Image 1.62c software. Images were combined in Adobe Photoshop 5.5 software.

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