Review

How do genes regulate simple behaviours? Understanding how different neurons in the vertebrate spinal cord are genetically specified

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Understanding how the vertebrate central nervous system develops and functions is a major goal of a large body of biological research. This research is driven both by intellectual curiosity about this amazing organ that coordinates our conscious and unconscious bodily processes, perceptions and actions and by the practical desire to develop effective treatments for people with spinal cord injuries or neurological diseases. In recent years, we have learnt an impressive amount about how the nerve cells that communicate with muscles, motoneurons, are made in a developing embryo and this knowledge has enabled researchers to grow motoneurons from stem cells. Building on the success of these studies, researchers have now started to unravel how most of the other nerve cells in the spinal cord are made and function. This review will describe what we currently know about spinal cord nerve cell development, concentrating on the largest category of nerve cells, which are called interneurons. I will then discuss how we can build and expand upon this knowledge base to elucidate the complete genetic programme that determines how different spinal cord nerve cells are made and connected up into neural circuits with particular functions.

Keywords: interneuron; spinal cord; transcription factor; zebrafish; Shh; BMP

1. INTRODUCTION

What is the most intriguing question of all is whether the brain is powerful enough to solve the problem of its own creation

(Eichele 1992)

Fifty-two years since the structure of DNA was elucidated and 140 years since Mendel first recognized the principle of genetic inheritance, the importance of DNA and the genetic code for making us who we are is almost universally recognized. We understand the genetic basis of several diseases and developmental disorders and we are learning more every day. Yet, even now, it is almost impossible to connect genes with behaviour. This is because there are many complex steps between the regulation of the specific genes that are turned on in particular cells and the behavioural repertoire that an animal becomes capable of and the behaviours that it engages in.

Despite this complexity, studies of two simple kinds of behaviour, movement and detection of specific stimuli like heat, touch and pain, have made considerable progress. Partly, this is because these behaviours can be studied in simpler vertebrates such as the lamprey, frog and zebrafish and partly it is because both of these behaviours are firmly rooted in the spinal cord. The spinal cord is the part of the vertebrate central nervous system that extends down the back inside the vertebral column. The spinal cord is a much simpler structure than the brain, and it is therefore much easier to study and understand.

Understanding how the spinal cord is made and functions is an ambitious and interesting goal in its own right, but it is also crucial if we are to develop effective treatments for people with spinal cord injuries or neurological diseases. If we can understand how particular neural circuits normally develop and function we can start to try and rebuild damaged circuits, replace particular nerves damaged by disease or injury, and hopefully finally understand why degenerative diseases often affect specific neural cell types. In addition, as we learn more about these basic behaviours our findings will also provide a foundation for studying more complex behaviours and neural functions.

To understand how the spinal cord becomes capable of controlling movements and perceiving different sensory inputs we need to understand how different cell types in the spinal cord are generated. The nervous system consists of two major types of cell: neurons and glia. In this review, I will concentrate on how genes regulate the development of different types of spinal cord neurons. Neurons are the cells that constitute the nerves in the body and they are responsible for gathering, consolidating and transmitting information within the nervous system. Neurons have a unique specialized cell morphology. They each extend a long protrusion called an axon that sends signals to other cells and they also extend numerous short processes called dendrites that branch out from the cell in a tree-like fashion and receive signals from other neurons. Several different classes of neurons exist in the spinal cord. Each of these has several characteristic properties

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which include which genes it expresses (turns on), its cell body size and shape, the length and direction of its axonal projection, its pattern of dendrites, its complement of specific ion channels (protein complexes that control ions entering and leaving the cell and which are important for electrical and chemical signalling within the neuron), the chemical signals (called neurotransmitters) that it uses, the chemical signals that it can detect (via neurotransmitter receptors) and its electrical activity during specific behaviours.

The neurons that we know most about are the neurons that communicate with muscles, which are called motoneurons. There are only a few different types of motoneuron and these can be easily identified in developing embryos and adults as they extend axons out of the central nervous system to distinct muscle territories. This has enabled researchers to correlate relatively easily the molecular phenotypes of specific motoneurons (which genes they express) with their morphologies and functions (which muscles they extend their axons to). We have learnt an amazing amount about how motoneurons are made and in particular which genes motoneurons express at different stages of their development and how this is regulated (for reviews see Jessell 2000; Shirasaki & Pfaff 2002; Lewis & Eisen 2003). Excitingly, these findings are comprehensive enough that they have enabled researchers to grow motoneurons from stem cells (Wichterle *et al.* 2002).

While considerable progress has been made in understanding motoneuron development and function, most of the neurons in the spinal cord and brain are interneurons. Interneurons are nerve cells whose cell body and axon resides entirely within the spinal cord and brain, which makes it much harder to distinguish different types of interneurons from one another. Until now, studies of interneurons have occurred within different Biological disciplines: Systems Neuroscientists have analysed interneuron physiology and function and Developmental Biologists have investigated how cells are instructed (specified) to make different types of interneurons. These approaches have traditionally used different criteria to identify distinct classes of interneurons. Developmental Biologists have, generally, classified interneurons on the basis of which genes they express, whereas Systems Neuroscientists have classified interneurons on the basis of their morphology, electrical activity and expression of specific neurotransmitters. Both groups of scientists also consider the position of interneurons within the spinal cord, but Systems Neuroscientists usually study adults whereas Developmental Biologists study embryos. Since many interneurons migrate after they are generated in the embryo, in most cases it is still unclear how the interneurons that Developmental Biologists study are related to the interneurons that Systems Neuroscientists study. However, recent progress has been made in this area as Developmental Biologists have started to study the morphology and functional characteristics of interneurons that express particular genes and Systems Neuroscientists have started to determine which genes different functional populations of interneurons express.

In this review, I will describe what we currently know about how different types of neurons are specified in the

vertebrate spinal cord and how these Developmental Biology studies are beginning to connect up with studies of neuronal physiology and function. I will concentrate on interneurons, but occasionally I will use examples from motoneuron development as our more extensive knowledge in this area often suggests mechanisms that may also be important for interneuron development. I will focus on how cells are specified along the dorsal–ventral axis of the spinal cord. Recent work has started to elucidate how distinct motoneuron subtypes are specified at different antero-posterior positions in the embryo (e.g. Dasen *et al.* 2003; Sockanathan *et al.* 2003; Lewis & Eisen 2004), but there is still very little information about the extent to which interneuron subtypes differ along this axis and the mechanisms that may be involved in this.

In the first part of this review (§2), I will describe the distinct molecular classes of interneurons that have been identified in the embryonic vertebrate spinal cord. I will also summarize the current state of knowledge about how specific signalling pathways regulate the formation of these cells. Homeodomain and basic helix-loop-helix (bHLH) transcription factors play a key role in the specification of different interneurons and I will discuss the regulation and function of these transcription factors in §3. In §4, I will discuss the degree to which a correspondence between molecularly distinct interneurons and interneurons with specific morphologies and functions has been established. Further progress in understanding mammalian spinal cord development is hindered by the sheer complexity of the mammalian nervous system. In this light, I will go on to discuss how we can utilize simpler vertebrates, in parallel with studies in mammals, to build and expand upon our existing knowledge base. Section 5 specifically discusses the use of zebrafish as a model organism for studying spinal cord development and function. Finally, in §6, I will discuss possible future directions in the quest to elucidate the complete genetic programme that regulates the formation and specific functions of spinal cord neural circuitry.

2. CELLS ARE PATTERNED ALONG THE DEVELOPING DORSAL–VENTRAL SPINAL CORD AXIS

(a) *Spinal cord cells in different dorsal–ventral positions express distinct combinations of transcription factors*

Over the last 10 years experiments by several researchers have identified at least 14 distinct populations of post-mitotic neurons in the embryonic chick and mouse spinal cord. Each of these neuronal populations forms in a particular dorsal–ventral position and usually expresses a unique combination of genes as soon as the cells become post-mitotic. The genes that researchers have concentrated on are those that encode transcription factors. Transcription factors are specialized proteins that bind DNA and regulate the expression of other genes. Each of these populations of post-mitotic neurons develops from a specific dorsal–ventral progenitor domain in the developing spinal cord that also expresses a unique combination of transcription factors (e.g. Briscoe *et al.* 2000;

figure 1*b,c* and table 1). These different progenitor domains are located in the most medial section of the spinal cord, which is called the ventricular zone (figure 1*b*). As cells stop proliferating in the early developing spinal cord, exit the cell cycle and become post-mitotic, they move away from the ventricular zone towards the lateral edges of the spinal cord and start to express different transcription factors (figure 1*b*; table 1). Studies so far suggest that as these cells differentiate, at least some of the neuronal characteristics that they acquire are determined by the transcription factors that they express during their development, and particularly the transcription factors that they express as soon as they become post-mitotic.

(b) The ventral spinal cord generates five cardinal classes of neurons

The ventral spinal cord primarily contains neurons that are involved in controlling movements (locomotor control) and relaying information about trunk and limb position (proprioception). These ventral neurons include motoneurons and four¹ cardinal classes of molecularly distinct interneurons (V0–V3; figure 1*b*). At least two of these interneuron populations can be further subdivided on the basis of transcription factor expression. For example, antibody staining distinguishes two spatially intermixed sub-populations of V2 cells: about half of V2 cells express Gata2 and Gata3, whereas the other half express Chx10 and Lhx3 (Karunaratne *et al.* 2002; Smith *et al.* 2002). These two sub-populations of cells have, therefore, been named V2a and V2b, respectively. Similarly, the p0 progenitor domain, which is the only domain in the spinal cord that expresses the transcription factor Dbx1 (figure 1*b,c*) generates Evx1 expressing cells ventrally (traditionally called V0 cells) and cells that do not express Evx1 dorsally (Pierani *et al.* 2001; Lanuza *et al.* 2004). Therefore, Lanuza and colleagues have suggested that these cells should be referred to as V0_D and V0_V interneurons. In addition, only about 50–60% of cells expressing *Evx1* also express Pax2, suggesting that there may also be different subsets of V0_V cells (Burrill *et al.* 1997). As the expression of more genes is characterized it is likely that further subdivisions of these ventral interneuron populations will be revealed. This may suggest that there are a greater number of cardinal classes of ventral interneurons than has been appreciated so far, but it could also reflect subsequent diversification and specialization of neuronal subtypes within a cardinal population. For example, while all somatic motoneurons develop from the pMN domain and initially express the same transcription factors, they then assume different subtype identities. Motoneurons that innervate different muscles start to express different transcription factors and often even acquire a distinct spatial organization within the spinal cord (Briscoe & Ericson 2001 and references therein). In the case of motoneurons, some aspects of this diversification are regulated by signals between different motoneuron cells and some are regulated by external signals, for example from the mesoderm adjacent to the spinal cord (Jessell 2000 and references therein). It is possible that both of these

mechanisms may also regulate diversification within cardinal classes of interneurons.

(c) The dorsal spinal cord generates nine cardinal classes of neurons

The dorsal spinal cord primarily contains neurons that are involved in processing and relaying sensory information from the peripheral nervous system of the trunk and limbs (e.g. figure 2). Initially, six distinct populations of post-mitotic cells form in the dorsal spinal cord (dI1–dI6). Three additional populations of cells are born later: dI1B cells are born in the dP1 domain and express the transcription factor Lhx2 but not Lhx9 (Lee *et al.* 1998) and dIL_A and dIL_B cells are generated in a salt and pepper fashion from a broad progenitor domain that probably encompasses dP4 and dP5 (Gowan *et al.* 2001; Muller *et al.* 2002). dIL_A cells express the transcription factors Pax2, Lhx1, Lhx5 and Gbx1 and dIL_B cells express Lmx1b and have smaller nuclei than dI4 and dI5 cells (Gross *et al.* 2002; Muller *et al.* 2002; Helms & Johnson 2003; John *et al.* 2005 and references therein; Caspary & Anderson 2003 and references therein). Understanding the development of different dorsal interneurons has been complicated by the considerable cell movements that some of these neurons undergo. dI1, dI1B, dI2, dI3, dI6 and at least a subset of dI5 interneurons migrate ventrally and dI4, dIL_A, dIL_B and maybe a subset of dI5 cells migrate laterally and/or dorsally (Gross *et al.* 2002; Muller *et al.* 2002; figure 2*a*; table 1). These cell movements and the similarity of gene expression between some of the different classes of dorsal neurons mean that in several cases the exact correlation between origin and final position is not yet known.

(d) Secreted signalling proteins establish different dorsal–ventral progenitor domains in the spinal cord

The first step in the specification of different spinal cord interneurons is the establishment of distinct dorsal–ventral spinal cord progenitor domains (figure 1*b*). A huge body of research suggests that these dorsal–ventral progenitor domains are established by two opposing gradients of secreted signalling proteins (figure 1*a*). Sonic Hedgehog (Shh) emanates from the notochord, a mesodermal rod of cells that underlies the ventral spinal cord and the floor plate, a specialized region comprising the most ventral part of the spinal cord (Echelard *et al.* 1993; Krauss *et al.* 1993; Roelink *et al.* 1994; figure 1*b*). Bone morphogenetic proteins (BMPs) emanate from the surface ectoderm and the dorsal spinal cord (Liem *et al.* 1995, 1997; Lee *et al.* 1998). Most researchers have concentrated either on how Shh signalling instructs cells to adopt (specifies) ventral fates or how BMP signalling specifies dorsal fates. However, while there is no convincing evidence that Shh signals normally influence the most dorsal fates in the spinal cord (Chiang *et al.* 1996; Wijgerde *et al.* 2002) the effects of BMP signalling may extend to the ventral spinal cord. In addition to specifying dorsal fates, BMP signals may normally act to restrict the expansion of ventral fates (figure 1*d*). For example, mouse embryos that lack notochord expression of a BMP antagonist, Noggin, have a reduced floorplate

Table 1. Characteristics of post-mitotic neurons. (A summary of current knowledge about the different post-mitotic neuronal populations in the spinal cord. More tentative conclusions are indicated with question marks. Key references are indicated in the right-hand column. Proprioception is the kinaesthetic sense of position and movement provides positional information about the limbs and body (i.e. the position of body parts with respect to each other). Nociception detects pain (both burning pain and sharp pain). Ipsilateral axons extend on the same side of the embryo as the cell body. Contralateral/commissural axons cross the midline and extend on the opposite side of the embryo to the cell body. Different laminae of the dorsal horn are shown in figure 2. Gene names given in the table are the official names according to the Mouse Genome Informatics web page (<http://www.informatics.jax.org/>). For previous and alternate gene names see table 2.)

post-mitotic interneuron population	progenitor domain origin	post-mitotic transcription factor expression	final position of cell body	axonal morphology	functional properties	key references
dI1 (called D1 in several earlier papers)	dP1 early	Lhx2, Lhx9, Barhl1, Barhl2, Pou4f1	Migrate ventrally and laterally to the deep dorsal horn.	Ascending commissural axons.	Form a subset of proprioceptor interneurons of the spino-cerebellar tracts.	Liem <i>et al.</i> (1997), Helms & Johnson (1998), Ben-Arie <i>et al.</i> (2000), Bermingham <i>et al.</i> (2001), Gross <i>et al.</i> (2002) and Saba <i>et al.</i> (2003)
dI1B	dP1 late	Lhx2 (not reported whether they express Barhl1 or Pou4f1)	Deep dorsal horn near, but distinct from, dI1 cells.		Probably involved in proprioception.	Lee <i>et al.</i> (1998)
dI2	dP2 early	Lhx1, Lhx5, Pou4f1, Foxd3	Migrate ventrally to the deep dorsal horn.	Ascending commissural interneurons.		Gowan <i>et al.</i> (2001) and Gross <i>et al.</i> (2002)
dI3 (called D2 in several earlier papers)	dP3 early	Isl1, Isl2, Pou4f1, Tlx3	Deep dorsal horn.	At least some may be ipsilateral.		Liem <i>et al.</i> (1997), Gross <i>et al.</i> (2002) and Helms <i>et al.</i> (2005)
dI4	dP4 early?	Lbx1h, Lhx1, Lhx5, Pax2	Laminae IV–V of dorsal horn?			Burrill <i>et al.</i> (1997) and Helms <i>et al.</i> (2005)
DIL _A	dP4/dP5 late?	Lbx1h, Lhx1, Lhx5, Pax2, Gbx1	Superficial laminae (I–III) of dorsal horn.		Mediate sensing of pain (nociception).	Burrill <i>et al.</i> (1997), Gross <i>et al.</i> (2002), Muller <i>et al.</i> (2002) and John <i>et al.</i> (2005)
dI5	dP5 early	Lbx1h, Pou4f1, Lmx1b, Tlx3, Tlx1, Phox2a (after migrate)	At least a subset migrate ventrally. Some cells might also migrate into the dorsal horn.			Muller <i>et al.</i> (2002), Qian <i>et al.</i> (2002) and Helms <i>et al.</i> (2005)
DIL _B	dP4/dP5 late?	Lbx1h, Pou4f1, Lmx1b, Tlx3, Prrxl1 (once in dorsal horn)	Superficial laminae (I–III) of dorsal horn.		Mediate sensing of pain (nociception).	Chen <i>et al.</i> (2001a,b), Gross <i>et al.</i> (2002), Muller <i>et al.</i> (2002), Qian <i>et al.</i> (2002) and Ding <i>et al.</i> (2004)
dI6	dP6 early	Lbx1h, Lhx1, Lhx5, Pax2	Migrate ventro-medially and settle in the ventral horn medial to motoneurons and close to floor plate.	At least most cells have contralateral ascending axons.		Pierani <i>et al.</i> (2001) and Muller <i>et al.</i> (2002)

(Continued.)

Table 1. (*Continued.*)

post-mitotic interneuron population	progenitor domain origin	post-mitotic transcription factor expression	final position of cell body	axonal morphology	functional properties	key references
V0	p0	V0 _V cells express Evx1 + Evx2. A subset of these cells also express Pax2. V0 _D cells are Evx1 negative.	Migrate ventro-medially and settle in the ventral horn medial to motoneurons and close to floor plate (lamina VIII).	Contralateral ascending axons. Descend to floorplate, cross midline and extend 1–4 segments. At least most of the cells contact motoneurons.	Involved in coordinating left–right alternation in locomotor activity. 70% of V0 neurons are inhibitory and 30% are excitatory.	Briscoe <i>et al.</i> (2000), Moran-Rivard <i>et al.</i> (2001), Pierani <i>et al.</i> (2001) and Lanuza <i>et al.</i> (2004)
V1	p1 early	En1, Pax2, Foxd3	Migrate laterally and settle in the ventral horn immediately dorsal to motoneuron cell bodies in lamina VI, VII and ventral lamina IX.	Ipsilateral ascending axons. Axons project ventrally and then extend rostrally for 1–2 segments. Renshaw cells extend both rostrally and caudally.	Renshaw cells all derive from V1 cells—but they are only 10% of V1 cells. Ia and Ib inhibitory interneurons probably also derive from V1 cells. All of these interneurons fine-tune motor behaviours.	Burrill <i>et al.</i> (1997), Ericson <i>et al.</i> (1997), Saueressig <i>et al.</i> (1999), Briscoe <i>et al.</i> (2000), Pierani <i>et al.</i> (2001), Goulding <i>et al.</i> (2002) and Sapir <i>et al.</i> (2004)
V2	p2 early	V2a cells express Chx10 + Lhx3. V2b cells express Gata2 + Gata 3. Medial V2b cells also express Tal1.	Ventral horn, dorsomedial to motoneuron cell bodies but ventral to V1 interneurons.	Ipsilateral axons that project for greater than 3–5 segments caudally and rostrally?		Ericson <i>et al.</i> (1997), Saueressig <i>et al.</i> (1999), Briscoe <i>et al.</i> (2000), Karunaratne <i>et al.</i> (2002) and Smith <i>et al.</i> (2002)
MN	pMN	Lhx3, Isl1, Hlxb9	Ventral horn.	Axon path is determined by MN subtype identity.	Synapse directly onto muscle cells.	Jessell (2000) and Shirasaki and Pfaff (2002)
V3	p3 early	Sim1				Briscoe <i>et al.</i> (1999)

Figure 1. Patterning of the mammalian spinal cord. (a) Schematic showing gradients of Hh and BMP signalling in the embryonic vertebrate spinal cord. BMP signalling is highest in the dorsal spinal cord (the part of the spinal cord closest to the back of the embryo) and Shh signalling is highest in the ventral spinal cord (the part of the spinal cord closest to the front or abdomen side of the embryo). In all cases dorsal is up and ventral is down. The dotted blue line indicates that the influence of BMP signalling may extend to the ventral spinal cord (see discussion in §2d). (b) Schematic cross-section through the embryonic mammalian spinal cord. The spinal cord is a tube of cells that runs along the trunk of the embryo. Progenitor domains are located medially in the ventricular zone and post-mitotic cells are located at the lateral edges of the spinal cord. The notochord is a rod of mesodermal cells that underlies the spinal cord. RP, roof plate; FP, floor plate; dP1–dP6, pMN and p1–p3 are different progenitor domains. dI1–dI6, MN and V0–V3 are different populations of post-mitotic neurons. (c) Schematic showing the transcription factors expressed in particular progenitor domains. N1 refers to, Neurog1. bHLH proteins are indicated by different shades of green, Class I homeodomain proteins are indicated by different shades of blue, Class II homeodomain proteins are indicated by different shades of red and dorsal homeodomain proteins that have not been shown to be class I proteins are indicated by different shades of orange. Proteins that share an expression boundary and cross-repress each other are vertically aligned. Nkx2.9 expression is not indicated but is identical to Nkx2.2 expression in the p3 domain. Neurog2 expression is not indicated but it partially overlaps the dorsal Neurog1 domain and the Ascl1 domain (dI2–dI5; Helms *et al.* 2005). Dbx1 expression extends slightly into the Pax7 domain (Muller *et al.* 2002). Pax7 expression is high in dP4–6 and lower more dorsally (indicated by shading). (d) A model of how BMP and Shh signalling may interact to specify dorsal and ventral spinal cord fates. BMPs, Shh and Noggin are all secreted signalling proteins. Ventrally expressed Shh induces ventral fates whereas dorsally expressed BMPs induce dorsal fates and repress ventral fates. The repression of ventral fates by BMPs is removed in the ventral spinal cord by ventrally expressed BMP inhibitors such as Noggin.

and a reduced number of ventral neurons in the caudal spinal cord (McMahon *et al.* 1998). Conversely, zebrafish embryos mutant for specific BMP proteins have expanded floorplate and ventral neuron populations (Barth *et al.* 1999; Nguyen *et al.* 2000), and when a different BMP inhibitor called Chordin is ectopically applied to the chick ventral spinal cord the floorplate expands (Patten & Placzek 2002). These results all suggest that both Shh signalling and repression of BMP signalling is required for ventral spinal cord fates to be specified correctly. Shh and BMP signalling pathways may even interact directly to control ventral spinal cord fates as *in vitro* studies

suggest both that BMP signalling can downregulate the response of cells to Shh signalling by acting on the Shh signalling pathway somewhere between cells receiving the Shh signal and regulating Shh target genes (Liem *et al.* 2000), and that the transcriptional effectors of these two pathways can physically associate with each other (Liu *et al.* 1998).

(i) Shh signals pattern the ventral spinal cord

As discussed above, a gradient of Shh signalling specifies distinct fates at different dorsal–ventral positions of the ventral spinal cord (figure 1a). Mouse embryos that lack Shh protein lose most ventral spinal

Figure 2. Migration and functions of dorsal interneurons. Adapted from Caspary & Anderson (2003) with permission from Nature Publishing Group (Copyright, 2003; <http://www.nature.com/>). (a) By embryonic day (E) 10, six post-mitotic dorsal interneuron populations are specified, each of which expresses a different combination of transcription factors. The dorsal interneuron (dI) subtypes dI1–dI3 and dI6 migrate ventrally, and dI4 and a subset of dI5 cells migrate laterally to populate the deep dorsal horn (laminae IV–V). Exact correlations between origin in the progenitor domain and position in the mature spinal cord are not known in most cases. Three additional populations of neurons are generated later, beginning at E12. dI1B cells express *Lmx1b* and settle deep in the dorsal horn near the earlier generated dI1 cells: both cell types are involved in proprioception. dI1A and dI1B cells are generated in a salt and pepper fashion and then migrate to the superficial laminae of the dorsal horn, where they mediate the sensing of pain and temperature. (b) Schematic of neuronal circuits in a mouse spinal cord at E18. The axons of peripheral nervous system sensory neurons project from the dorsal root ganglia to specific laminae in the dorsal horn and to the periphery. Pain and temperature are sensed by nociceptive neurons (red), and the messages are conveyed to laminae I and II. Touch is mediated by mechanoreceptors (green) in the periphery, which connect to laminae III–V. Proprioception, which is the kinesthetic sense of position and movement that provides positional information about the limbs and body, is mediated by the sensory neurons that project through the dorsal horn to the ventrally located motor neurons (shown in blue). The motor neurons also connect directly back to peripheral muscles to drive movement (pink). Renshaw cells (shown in yellow and indicated by R) receive excitatory input from motor neuron collaterals and in turn project back onto and inhibit motor neurons. Roman numerals indicate the laminae of the dorsal horn.

cord neurons showing that Shh signalling is required for ventral spinal cord patterning (Chiang *et al.* 1996). In addition, both *in vitro* and *in vivo* experiments have shown that Shh signalling is sufficient to induce ventral neurons, with high concentrations of Shh signalling specifying the most ventral cell types and lower levels of Shh signalling specifying more dorsal fates (Marti *et al.* 1995; Roelink *et al.* 1995; Ericson *et al.* 1996, 1997; Briscoe *et al.* 1999, 2000). Finally, two different types of *in vivo* experiment demonstrate that Shh signalling acts directly to specify ventral spinal cord fates. Firstly,

ectopic activation of the Shh signalling pathway cell-autonomously induces ventral neuronal fates (Hynes *et al.* 2000) and secondly, ventral spinal cord cells which can no longer respond to Shh signals, because they have mutations in components of the Shh signalling pathway, no longer develop into ventral neurons but instead acquire dorsal interneuron characteristics (Briscoe *et al.* 2001; Wijgerde *et al.* 2002).

While Shh signalling is crucial for patterning the ventral spinal cord, it is probably not the only signalling pathway that is involved in this process. For example,

even though different levels of Shh signalling can specify all of the different ventral neurons, Shh signalling is not required for the generation of the most dorsal of these cell types: V0 and V1 interneurons. These interneurons can also be specified by retinoids, which emanate from the paraxial mesoderm and the spinal cord and act in parallel to Shh signalling (Pierani *et al.* 2001). However, more recent results suggest that even though Shh signalling is not required for V0 and V1 interneuron specification some Hedgehog (Hh) signalling is required for at least V1 cells to form. Shh is only one member of a family of related Hh proteins in vertebrates, all of which signal through the same signalling pathway. Cells that are mutant for Smoothed, an essential downstream component of the Hh signalling pathway, cannot generate MNs or V1–V3 interneurons. In contrast, very occasionally mutant cells can generate V0 cells, but only in the most ventral part of the SC (Wijgerde *et al.* 2002). These results suggest that at least some V0 neurons can develop in the complete absence of Hh signalling, but that V1 neurons require Hh signalling for their specification. The only other Hh protein that is expressed in the vicinity of the spinal cord is Indian hedgehog (Ihh), which is expressed in the gut endoderm underlying the spinal cord. Therefore, it is likely that in Shh mutants, Ihh provides enough Hh signalling in the spinal cord for V1 interneurons still to be generated (Wijgerde *et al.* 2002).

(ii) *Shh signalling removes the repression of ventral fates*

In what is a surprising twist to this story, more recent experiments have suggested that with the exception of the floor plate and p3 domain, the primary function of Hh signalling in the spinal cord is not to induce different fates directly, but rather to remove an active repression of ventral fates. Gli3 is a zinc finger transcription factor that represses transcription of Hh signalling target genes in the absence of Hh signals but is converted to a transcriptional activator by Hh signalling (Jacob & Briscoe 2003 and references therein). Interestingly, mouse embryos that lack both Gli3 and Shh signalling recover several, although not all, of the ventral fates lost in Shh mutant embryos (Litington & Chiang 2000) as do mouse embryos that lack both Gli3 and Smoothed (Wijgerde *et al.* 2002) and mouse embryos that lack Gli3 and the related transcription factor Gli2 (the main downstream transcriptional activator of Hh signalling; Lei *et al.* 2004). All of these double mutant embryos lack floor plate and V3 interneurons and have a reduced number of motoneurons, but unlike embryos that lack all Hh signalling but still have Gli3 activity, these embryos generate V2, V1 and V0 interneurons. These results suggest that Hh signals act in a Gli3 independent manner to induce floor plate and V3 interneurons, but that the main mechanism by which Hh signalling specifies V2, V1 and V0 interneurons is by removing Gli3 repressive activity. These findings also suggest that there is probably a Hh-independent mechanism for specifying all but the most ventral neuronal fates in the ventral spinal cord. This Hh-independent mechanism may differ in its efficacy along the rostral–caudal axis as there are subtle rostral–caudal differences in the double

mutant phenotypes. For example, in *Gli3*; *Smo* double mutant embryos less V2 interneurons and motoneurons form in the posterior spinal cord than the anterior spinal cord (Wijgerde *et al.* 2002). It will be interesting to determine if BMP signals are required for the specification of V0, V1 or V2 neurons in the absence of Hh signalling and Gli3 activity. However, testing this is not trivial as it will probably require removing BMP signals at the earliest stages of neural development, which is hard to do without disturbing embryonic development more generally (see §2d(iv)).

Interestingly, the spatial organization of the ventral neurons that form in embryos that lack all Hh signalling and Gli3 activity is no longer as distinct as in wild-type embryos (Wijgerde *et al.* 2002; Lei *et al.* 2004). For example, in *Gli3*; *Smo* double mutant embryos V1 and V2 interneurons and motoneurons are intermingled over a wide area of the ventral spinal cord (Wijgerde *et al.* 2002). This is one of the most surprising results from these double mutant analyses as it suggests that the primary function of Hh signalling and Gli3 activity in the ventral-intermediate spinal cord is the spatial organization of neurons rather than the specification of different neuronal fates. The balance between activation of Hh signalling target genes by Shh signalling and repression of Hh target genes by the repressive form of Gli3 is probably crucial in this process as mouse embryos that just lack Gli3 also have a small amount of intermingling between adjacent interneuron populations (V2 and V1; V1 and V0) that is not normally seen in wild-type embryos (Persson *et al.* 2002). The intermingling of ventral interneurons in embryos that lack Hh signalling and Gli3 activity is prefigured in the ventricular zone of the spinal cord by the intermingling of cells expressing transcription factors that are normally expressed in different progenitor domains (Persson *et al.* 2002; Wijgerde *et al.* 2002; Lei *et al.* 2004). This suggests that the primary function of the dorsal–ventral balance between Hh signalling and Gli3 repressive activity is to spatially regulate progenitor domain transcription factor expression so that distinct dorsal–ventral progenitor domains are generated. This new model of ventral spinal cord patterning raises many interesting questions including how and when this function of Hh signalling in spatially organizing neurons evolved, if and why it is important for different neurons to be generated in precise domains and what other signals or mechanisms can specify different neuronal fates in the ventral spinal cord.

(iii) *The roof plate is required for the most dorsal spinal cord fates*

The roof plate, which is a specialized group of cells at the most dorsal point of the spinal cord, has a crucial role in patterning dorsal interneurons. If the roof plate is genetically ablated in embryos the three most dorsal progenitor domains (d1–d3) and their associated post-mitotic interneurons (dI1–dI3) are lost (Lee *et al.* 2000). The roof plate expresses several secreted signalling proteins, including members of the Wnt and BMP families. For example, in mouse embryos the roof plate expresses Wnt 1, Wnt 3a, Bmp6, Bmp7 and Gdf7 (Lee *et al.* 1998; Wine-Lee *et al.* 2004), whereas in chick embryos Wnt1, Wnt3a, Bmp4, Bmp5, Bmp7,

Gdf6/7 are expressed in the roof plate (Liem *et al.* 1995, 1997; Lee *et al.* 1998). BMP proteins belong to the TGF β super family of proteins and two other members of this family, Dorsalin1 (DSL-1) and Activin B are also expressed in the chick roof plate (Liem *et al.* 1997). In both chick and mouse several of these signalling molecules are also expressed in cells adjacent to the roof plate in the dorsal spinal cord.

(iv) *BMP signals are required to specify dorsal interneurons*

Several studies have investigated which signalling proteins are important for specifying different dorsal fates in the spinal cord. BMP signals have been shown in a variety of ways to be good candidates for this process. For example, BMP signalling can induce dorsal interneurons in neural plate explants, ectopic BMP signals can induce dorsal neurons *in vivo* and BMP inhibitors can prevent roof plate from inducing dorsal interneurons in neural plate explants (Liem *et al.* 1997; Lee *et al.* 1998; Timmer *et al.* 2002; Liu *et al.* 2004). Establishing an *in vivo* requirement for BMP signalling in dorsal–ventral patterning of the spinal cord has historically been more difficult as most BMP signals have essential functions earlier in development. Mouse embryos that lack Bmp2, Bmp4 or the BMP receptors Bmpr1a or Bmpr2 die too early for neural development to be analysed (Caspary & Anderson 2003 and references therein). Mouse embryos mutant for Bmp7 or Bmpr1B survive long enough to establish that they do not have any defects in dorsal interneuron specification (Caspary & Anderson 2003 and references therein) but this may be due to functional redundancy with other BMP proteins or receptors. In contrast, mouse embryos that lack the BMP family member Gdf7 have a substantial reduction of dI1 interneurons, although these cells initially appear to form normally (Lee *et al.* 1998), and zebrafish embryos mutant for different components of the BMP signalling pathway have defects in the specification of different spinal cord interneurons (Barth *et al.* 1999; Nguyen *et al.* 2000; see also discussion in Lewis & Eisen 2003). More recent experiments have established a clear requirement for BMP signalling in the specification of the most dorsal three populations of interneurons in birds and mammals. Mouse embryos mutant for Bmpr1b that also lack Bmpr1a specifically in the spinal cord have a complete loss of dI1 neurons and a substantial reduction of dI2 neurons (Wine-Lee *et al.* 2004). In addition, chick embryos where BMP signalling has been substantially reduced in the spinal cord, either by ectopic expression of a BMP antagonist, Noggin, or by knocking down the function of a downstream component of BMP signalling, Smad4, lose dI1 neurons and have reduced numbers of dI2 and dI3 neurons (Chesnutt *et al.* 2004).

It is still not completely clear whether different dorsal fates are specified simply by a gradient of BMP signalling or whether there are also important qualitative differences between different BMP or TGF β signals. *In vitro* experiments have suggested that different concentrations of BMP signalling induce different fates, but they have also suggested that there may be qualitative differences in the signalling activities

of different TGF β proteins expressed in the dorsal spinal cord (Liem *et al.* 1997). In agreement with this, Timmer and colleagues have recently shown that ectopic expression of a constitutive active Activin receptor selectively increases the number of dI3 neurons. Interestingly, no other cell population appeared to be reduced in these experiments and the same result was generated at a variety of concentrations of Activin signalling, suggesting that Activin ligands do not function in a graded manner in the dorsal spinal cord (Timmer *et al.* 2005). Most of the experiments that have tried to address whether BMP signalling is required for dorsal specification have used methods that remove or reduce all BMP signalling, which makes it difficult to draw any conclusions about qualitative differences between BMP proteins or BMP receptors *in vivo*. However, low level expression of an activated BMP receptor *in vivo* induces dI3 neurons and higher levels induce dI1 neurons (Timmer *et al.* 2002), suggesting that different concentrations of BMP signalling are sufficient to specify different dorsal fates *in vivo*. *In vitro* experiments have also suggested that the time at which cells receive BMP signals is important for determining which fates are specified (Liem *et al.* 1997; Liu *et al.* 2004). Which BMP receptor is activated at a specific time may also determine whether cells respond to BMP signals by acquiring specific fates, proliferating, differentiating, or even dying (Panchision *et al.* 2001).

So far, there is no conclusive evidence that BMP signals are required for dI4–dI6 specification in birds and mammals, suggesting that these cells may be specified by a different mechanism. However, as mentioned above in §2d, there is evidence that BMP signals influence patterning throughout the dorsal–ventral extent of the spinal cord in both zebrafish and mouse embryos (McMahon *et al.* 1998; Barth *et al.* 1999; Nguyen *et al.* 2000). The discrepancy between these different results may be due to the fact that the studies that demonstrated a direct requirement for BMP signalling in specifying dP1–dP3 progenitor domains specifically knocked down BMP signalling in the spinal cord (Chesnutt *et al.* 2004; Wine-Lee *et al.* 2004). The pleiotropic effects of BMP signalling and its requirement during early embryogenesis make it very difficult to analyse the effects of complete loss of BMP signalling on dorsal–ventral spinal cord specification and both of these studies left earlier BMP signalling intact. In contrast, when BMP-expressing surface ectoderm and dorsal spinal cord was ablated at a slightly earlier stage in chick embryos the floorplate expanded (Patten & Placzek 2002). Therefore, it is still possible that BMP signalling at neural plate stages or during neurulation is required to correctly specify dP4–dP6 and maybe even more ventral fates in birds and mammals.

Interestingly, while Shh signalling is not required to specify any dorsal interneuron fates, Gli3 repressor activity (which presumably represses Shh target genes) is required for the correct number of dI6, dI5 and dI4 neurons to be generated. Gli3 mutants have a dorsal expansion of the p0, p1 and dP6 progenitor domains and excess V0, V1 and dP6 neurons form at the expense of dI5 and dI4 neurons (Persson *et al.* 2002). If

Gli3 is replaced with a form of the protein that acts exclusively as a transcriptional repressor these phenotypes are rescued (Persson *et al.* 2002), demonstrating that it is specifically Gli3 repressor activity that is required in the intermediate spinal cord to prevent ventral neuronal fates from expanding too far dorsal. Given that the transcriptional effectors of BMP signalling (Smad proteins) can physically interact with Gli proteins *in vitro* (Liu *et al.* 1998), it will be interesting to determine if BMP signalling is directly involved in this regulation of intermediate spinal cord fates by Gli3.

(v) *The role of Wnt signals in dorsal spinal cord development*

Wnt signals are also important for correct development of different dorsal interneuron populations. In recent years, there has been a debate about whether Wnt signals are required for cell proliferation in the dorsal spinal cord or whether they have a direct role in fate specification. The expression of Wnt1a and Wnt3a in the dorsal spinal cord is probably regulated by BMP signalling (Marcelle *et al.* 1997; Panchision *et al.* 2001; Chesnutt *et al.* 2004), suggesting that BMPs might specify different dorsal fates, at least in part, by inducing Wnt1 and Wnt3a expression. Mouse embryos mutant for either Wnt1 or Wnt3a form normal numbers of dorsal interneurons, but double mutant embryos that lack both Wnt1 and Wnt3a have drastically reduced numbers of dI1 and dI3 neurons and increased numbers of dI4 neurons, suggesting that Wnt1 and Wnt3a may have redundant functions in dorsal spinal cord patterning (Muroyama *et al.* 2002). In addition, *in vitro* experiments show that Wnt3a can induce dI1 and dI3 neurons in neural explants (Muroyama *et al.* 2002). In both of these examples Wnt signalling appears to be independent, or downstream, of BMP signalling as BMPs are expressed normally in Wnt1; Wnt3a double mutant embryos and co-expression of a BMP inhibitor Noggin does not interfere with the ability of Wnt3a to induce dI1 and dI3 neurons *in vitro* (Muroyama *et al.* 2002). In contrast, previous studies have suggested that Wnt signals control proliferation in the spinal cord (Dickinson *et al.* 1994; Megason & McMahon 2002) and a recent study suggests that ectopic expression of Wnt3a is not sufficient to rescue the loss of dI1 neurons caused by a reduction in BMP signalling (Chesnutt *et al.* 2004). These results suggest that Wnt signalling is required for proliferation, rather than specification, of dorsal interneurons. In addition, experiments where BMP signalling was activated cell autonomously with constitutive active receptors or downregulated cell autonomously by knocking down a downstream component of BMP signalling suggest that BMP signals specify dI1, dI2 and dI3 fates directly (Timmer *et al.* 2002; Chesnutt *et al.* 2004). One possible explanation that would partly explain these different observations would be if Wnt signals primarily regulate cell proliferation but there is specificity between particular Wnts and the progenitor domains that they regulate. A variety of different Wnt signalling proteins, receptors and secreted inhibitors are expressed in different dorsal–ventral domains in the spinal cord, suggesting that regulation of Wnt signalling is quite complex and that different Wnts

may regulate the proliferation of specific cell types (Chesnutt *et al.* 2004 and references therein). Consistent with this idea, Wnt7a and the Wnt receptors Frizzled2 and Frizzled7, which are normally expressed in the intermediate spinal cord, are repressed by ectopic expression of activated BMP receptors and expand dorsally when BMP signalling is inhibited (Chesnutt *et al.* 2004). However, the ability of Wnt3a to induce dI1 and dI3 neurons *in vitro* (Muroyama *et al.* 2002) suggests that Wnt signals may also be able to specify particular interneurons in certain circumstances.

3. DIFFERENT COMBINATIONS OF HOMEODOMAIN AND bHLH TRANSCRIPTION FACTORS SPECIFY DISTINCT POPULATIONS OF SPINAL CORD INTERNEURONS

In §2, I discussed how several secreted signalling proteins specify distinct dorsal–ventral progenitor domains in the developing spinal cord, each of which generates a particular cardinal class of post-mitotic neurons. As mentioned in §2a, each of these progenitor domains expresses a particular combination of transcription factors, as do the post-mitotic cells that derive from these domains. In this section, I will discuss the regulation and function of these different transcription factors.

(a) *Shh signalling regulates transcription factor expression in ventral spinal cord progenitor domains*

As discussed in §2d, a gradient of Shh signalling is crucial for the correct specification of ventral progenitor domains in the vertebrate spinal cord. A variety of experiments suggest that Shh establishes distinct ventral progenitor domains by regulating the expression of progenitor domain transcription factors. With the exception of Olig2, which is a member of a family of proteins that contain a basic bHLH domain, all of these transcription factors contain a particular DNA binding domain called a homeodomain (figure 1c). These transcription factors can be divided into two groups: class I transcription factors are repressed by Shh signalling whereas class II transcription factors require Shh signalling for their expression. Different threshold concentrations of Shh signalling repress specific class I transcription factors and induce specific class II transcription factors (Briscoe *et al.* 2000; Briscoe & Ericson 2001 and references therein; figure 3). For example, Irx3 is expressed more ventrally than Dbx2 (figure 1c) and this correlates with Dbx2 expression being repressed at a lower concentration of Shh signalling than Irx3. Conversely, Nkx6.1 is expressed more dorsally than Nkx2.2 (figure 1c) and Nkx6.1 expression is induced at a lower concentration of Shh signalling than Nkx2.2. As a result of these differential responses to Shh signalling each ventral progenitor domain ends up expressing a unique combination of transcription factors (figure 1c). In addition, the boundary of each ventral progenitor domain is demarcated by the ventral limit of expression of a class I transcription factor and the dorsal limit of expression of a class II transcription factor (figures 1c

Figure 3. Shh signalling specifies distinct neurons by regulating the expression of different transcription factors. Adapted from Briscoe & Ericson 2001 with permission from Elsevier (Copyright, 2001). (a) Different concentrations of Shh signalling repress expression of specific class I proteins (e.g. Pax6 and Dbx2) and induce expression of specific class II proteins (e.g. Nkx2.2 and Nkx6.1) in the ventral spinal cord. (b) Cross-repressive interactions between class I and class II proteins with a common expression boundary refine and maintain progenitor domains. For example cells that express Nkx2.2 repress Pax6 and vice versa; cells that express Nkx6.1 repress Dbx2 and vice versa. (c) The particular combination of class I and class II proteins expressed within individual progenitor domains controls the fate of differentiating neurons.

and 3). In all cases examined so far, when the ventral limit of expression of a class I transcription factor corresponds to the dorsal limit of expression of a class II transcription factor, those two transcription factors cross-repress each other (Briscoe *et al.* 2000; figure 3). For example, loss of Pax6 results in the dorsal expansion of Nkx2.2 expression, ectopic expression of Pax6 can repress Nkx2.2 expression and ectopic expression of Nkx2.2 can repress Pax6 expression (Ericson *et al.* 1997; Briscoe *et al.* 2000). Cross-repressive interactions have also been shown for Irx3 and Olig2 (Mizuguchi *et al.* 2001; Novitsch *et al.* 2001; Lewis *et al.* 2005), Dbx2 and Nkx6.1 (Briscoe *et al.* 2000; Sander *et al.* 2000), and Dbx1 and Nkx6.2 (Vallstedt *et al.* 2001), although in the latter case only a subset of p1 domain cells express Dbx1 in the absence of Nkx6.2, suggesting that additional proteins can also inhibit ventral expansion of Dbx1 expression (Vallstedt *et al.* 2001). Interestingly, most of these class I and class II transcription factors act directly as transcriptional repressors (Muhr *et al.* 2001) suggesting that the specific identity of each progenitor domain may be achieved by suppressing all other possible fates.

(b) Dorsal progenitor domains differ in their expression of bHLH transcription factors

In a similar manner to the ventral spinal cord, different progenitor domains in the dorsal spinal cord express particular transcription factors. Interestingly, the most dorsal progenitor domains (dP1–dP3) can be distinguished by their differential expression of bHLH

Table 2. Official mouse gene names. (Official gene names according to the Mouse Genome Informatics web page (<http://www.informatics.jax.org/>) are given in the left-hand column and previously used names are given in the right-hand column.)

official mouse gene name	previous and alternative gene names
<i>Ascl1</i>	<i>Mash1</i> (chicken gene called <i>Cash1</i>)
<i>Atoh1</i>	<i>Math1</i> (chicken gene called <i>Cath1</i>)
<i>Barhl2</i>	<i>Mbh1</i>
<i>Hlxb9</i>	<i>MNR2</i>
<i>Isl1</i>	<i>Islet1</i>
<i>Isl2</i>	<i>Islet2</i>
<i>Lbx1h</i>	<i>Lbx1</i>
<i>Lhx1</i>	<i>Lim1</i>
<i>Lhx2</i>	<i>Lh2a</i>
<i>Lhx3</i>	<i>Lim3</i>
<i>Lhx5</i>	<i>Lim2</i>
<i>Lhx9</i>	<i>Lh2b</i>
<i>Neurog1</i>	<i>Ngn1</i>
<i>Neurog2</i>	<i>Ngn2</i>
<i>Pou4f1</i>	<i>Brn3a</i>
<i>Prrxl1</i>	<i>Drg11</i>
<i>Smo</i> (<i>Smoothened</i> homolog (Drosophila))	<i>Smoothened</i>
<i>Tal1</i>	<i>SCL</i>
<i>Tlx3</i>	<i>Rnx</i>

proteins (figure 1c). This was initially surprising, as bHLH proteins have well established general roles in inducing neurogenesis and in the ventral spinal cord most progenitor cells share expression of bHLH proteins Neurog1 (previously called Ngn1) and Neurog2 (previously called Ngn2), although a few express Ascl1 (previously called Cash1 in chicks and Mash1 in mice; Sommer *et al.* 1996; Gowan *et al.* 2001; Mizuguchi *et al.* 2001; Scardigli *et al.* 2001; Parras *et al.* 2002; table 2).

Despite these differences in the type of transcription factor that demarcates specific domains, in the dorsal spinal cord transcription factors in neighbouring progenitor domains repress each other's expression just like in the ventral spinal cord. For example, in mouse embryos mutant for either *Gsh2* or *Ascl1* Neurog1 expression expands ventrally and ectopic expression of Ascl1 or *Gsh2* can repress Neurog1 expression (Helms *et al.* 2005; Kriks *et al.* 2005). In *Atoh1* mutants Neurog1 and Neurog2 expression extends dorsally and in *Neurog1*; *Neurog2* double mutants *Atoh1* expression extends ventrally (Gowan *et al.* 2001). Likewise, ectopic expression of *Atoh1* (previously called Math1 in mice and Cath1 in chicks) in chick spinal cords can repress Neurog1 expression and ectopic expression of Neurog1 in chick spinal cords can repress *Atoh1* and *Ascl1* expression (Gowan *et al.* 2001; Kriks *et al.* 2005).

(c) Progenitor domain transcription factors determine the fates of post-mitotic cells

In all of the cases examined so far, the particular combination of transcription factors expressed by a progenitor domain is sufficient and required for determining the post-mitotic identity of the cells

generated from that domain. The primary role of progenitor domain transcription factors seems to be to control the identity of neurons generated by the domain, including the combination of transcription factors that the cells express post-mitotically. For example, in the ventral spinal cord the only difference identified so far between the p1 and p0 progenitor domains is the expression of Dbx1 in the p0 domain (figure 1c). In mouse embryos that lack Dbx1 at least most V_{0D} cells are respecified as dI6 neurons (Lanuza *et al.* 2004) and cells in the V_{0V} domain give rise to interneurons that exhibit many, although not all, of the features of V1 interneurons (Pierani *et al.* 2001). Conversely, ectopic expression of Dbx1 in chick embryos leads to an expansion of V_{0V} interneurons and a decrease in V1 interneurons (Pierani *et al.* 2001). Similarly, Nkx2.2 expression is required for the specification of V3 interneurons and ectopic expression of Nkx2.2 in the pMN progenitor domain causes ectopic production of V3 interneurons (Briscoe *et al.* 1999; Briscoe *et al.* 2000). Ectopic expression of Irx3 also prevents cells that would otherwise develop as motoneurons from adopting this fate choice—in this case they develop as V2 interneurons (Briscoe *et al.* 2000). Likewise, when Olig2 is ectopically expressed, Irx3 expression is downregulated and this correlates with a dorsal expansion of motoneurons (Novitsch *et al.* 2001).

As mentioned above, the discovery that the most dorsal progenitor domains are distinguished by differential expression of bHLH transcription factors was initially surprising as bHLH proteins were thought to be general neurogenic factors. However, when the functions of these bHLH transcription factors were examined they were found to play crucial roles in specifying the post-mitotic cells that are generated by these dorsal domains. For example, Olig3 is expressed in the dP1–dP3 domains (Ding *et al.* 2005; Muller *et al.* 2005; figure 1c) and Olig3 mutant mice lack dI2 and dI3 neurons and have about a 50% reduction of dI1 neurons (Muller *et al.* 2005). Atoh1 is exclusively expressed by the progenitor domain dP1 which generates dI1 neurons and Atoh1 is also required for these neurons to develop: loss of Atoh1 in mouse embryos correlates with a loss of dI1 neurons and ectopic expression of Atoh1 in the chick dorsal spinal cord results in an increased number of dI1 neurons (Helms & Johnson 1998; Ben-Arie *et al.* 2000; Bermingham *et al.* 2001; Gowan *et al.* 2001; Nakada *et al.* 2004). However, when Atoh1 expands ventrally in *Neurog1*; *Neurog2* double mutant mouse embryos no obvious expansion of dI1 cells is seen (Gowan *et al.* 2001), suggesting that Atoh1 is not always sufficient to specify the dI1 fate. In a similar manner, the dP2 progenitor domain expresses *Neurog1* and *Neurog2* and gives rise to dI2 neurons and this population of neurons is reduced in *Neurog1* mutants and lost completely in *Neurog1*; *Neurog2* double mutants (Gowan *et al.* 2001). Interestingly *Ascl1*, which is normally expressed in the presumed dP3, dP4 and dP5 progenitor domains, can specify dI3 and dI5 neurons but not dI4 neurons: ectopic expression of *Ascl1* in chick embryos increases the number of dI3 and dI5 neurons at the expense of dI2 and dI4/6 neurons (the

markers used could not distinguish between dI4 and dI6 neurons; Nakada *et al.* 2004; Helms *et al.* 2005; Kriks *et al.* 2005) and *Ascl1* mutant mice have about a 70% reduction of dI3 neurons, a complete loss of dI5 neurons and a concomitant increase in dI2 and dI4/6 neurons (Nakada *et al.* 2004; Helms *et al.* 2005; Kriks *et al.* 2005; but see also Guillemot *et al.* 1993). Consistent with these results, recombination-based lineage tracing shows that only a small subset of dI4 neurons are generated from *Ascl1*-expressing cells (Helms *et al.* 2005), although it is possible that larger numbers of dI4 neurons express low levels of *Ascl1* that were not detected by this method.

Given the dual roles of at least some bHLH proteins in neurogenesis and neural specification, recent experiments that show that the ability of *Ascl1* to promote neuronal differentiation is conferred by a different region of the protein to the region that confers its ability to ectopically induce dI3 neurons are particularly interesting. Helix 1 of the *Ascl1* HLH domain is required for promoting neuronal differentiation, and is sufficient to convert a non-neural bHLH protein such as the muscle-specific transcription factor *MyoD* into a neurogenic protein, whereas Helix 2 of the *Ascl1* HLH domain is required for *Ascl1* to specify dI3 neurons (Nakada *et al.* 2004). It will be interesting to see if other bHLH proteins that appear to specify specific types of interneurons also have distinct neuronal differentiation and interneuron specification domains.

There is some evidence that the bHLH proteins *Neurog1*, *Neurog2* and *Ascl1* may also have a role in specifying particular cell types in the ventral spinal cord, where they have historically been assumed to be general neurogenic factors. In wild-type embryos, *Ascl1* is expressed in the p2 domain and loss of *Ascl1* leads to a reduction of V2 neurons (Parras *et al.* 2002). In contrast, *Neurog2* is expressed more broadly in the ventral spinal cord and loss of *Neurog2* leads to a reduction of motoneurons, and V1 and V3 interneurons (Parras *et al.* 2002). In some elegant experiments, Parras and colleagues replaced *Neurog2* coding sequence with that of *Ascl1* and vice versa, so that the genes that encode for these two transcription factors were each expressed at the normal time and place of the other gene. Embryos where *Ascl1* was inserted into the *Neurog2* locus generated motoneurons and V2 neurons, but ectopic V2 (Chx10+; Islet−) cells were observed in the MN domain even though *Irx3* expression was not affected. Ectopic V2 cells were also observed in embryos heterozygous for the normal *Neurog2* allele and *Ascl1* inserted into the *Neurog2* locus, showing that the expression of *Ascl1* in the pMN domain has a dominant effect. In contrast, when *Neurog2* was inserted into the *Ascl1* locus there was a reduction of V2 cells and there was no evidence that V2 cells translocated to motoneurons or V1 interneurons, suggesting that *Neurog2* is unable to promote neurogenesis in the p2 domain (Parras *et al.* 2002). Similarly, *in vitro* experiments suggest that *Neurog2* and *NeuroM* can cooperate with *Isl1* and *Lhx3* to promote MN development but that *Ascl1* cannot (Lee & Pfaff 2003).

Taken together, these results suggest that both bHLH and homeodomain transcription factors

are important for specifying distinct populations of post-mitotic cells in the spinal cord. How the specification of neuronal subtype identity is integrated with the regulation of neurogenesis is still not understood for most neurons. Analysis of the motoneuron domain suggests that *Olig2* may induce expression of *Neurog1* (Novitch *et al.* 2001; but also see Mizuguchi *et al.* 2001) so it is possible that progenitor domain transcription factors are at least partially responsible for imposing the restricted pattern of neurogenic genes in the ventral spinal cord. Regulation of neurogenic genes may also be important in determining the transition between neural specification and glial specification in a particular progenitor domain (Kessaris *et al.* 2001 and references therein).

(d) Post-mitotic transcription factors can specify neuronal identity or regulate specific neuronal characteristics

Some post-mitotically expressed transcription factors also control the cell-type identities of the population of cells that express them. This suggests that there is a critical post-mitotic time window during which the differentiation programme of post-mitotic neurons can still be altered. For example, ectopic expression of *Hlxb9* (previously called *MNR2*) induces ectopic motoneurons without altering the expression of class I and class II progenitor domain transcription factors (Tanabe *et al.* 1998). Similarly, when *Evx1* is inactivated in mouse embryos, the interneurons that normally express *Evx1* appear to be respecified as V1 interneurons despite their previous expression of p0 transcription factors: their cell bodies migrate to the same position as V1 interneurons, they lose expression of *Evx2*, turn on expression of *Engrailed 1* (*En1*) and their axons follow a similar path to those of V1 interneurons (Saueressig *et al.* 1999; Moran-Rivard *et al.* 2001). Likewise, when *Lbx1h* is inactivated in mouse embryos dI4 and dI5 neurons appear to be mis-specified as dI2 and dI3 neurons, respectively, and dI6 neurons migrate like dI4 cells (Gross *et al.* 2002; Kruger *et al.* 2002; Muller *et al.* 2002).

In contrast, other post-mitotic transcription factors control much more specific aspects of neuronal development. For example, *Lhx1* (previously called *Lim1*) is required for the correct axon pathfinding of *LMC_L* motoneurons. In the absence of *Lhx1*, *LMC_L* motoneurons are specified normally but their axons innervate both the dorsal and ventral halves of the limb mesenchyme, whereas normally they only innervate the dorsal limb (Kania *et al.* 2000). In a similar manner, the post-mitotic transcription factor *Barhl2*, which is directly downstream of the dP1 progenitor domain transcription factor *Atoh1*, is required for the commissural axon trajectory of dII cells: the axons of dII cells that ectopically express a dominant negative form of *Barhl2* no longer cross the midline and dorsal cells that ectopically express *Barhl2* acquire a commissural axon trajectory (Saba *et al.* 2003, 2005). *En1* has an even more subtle function. *En1* is expressed by V1 neurons, which are a class of interneurons that normally communicate with motoneurons. If the *En1* gene is inactivated in mouse embryos, the early development of V1 interneurons appears to be completely normal

but the interneurons have defects in axon pathfinding and fasciculation within the ventrolateral funiculus (VLF; Saueressig *et al.* 1999) and they no longer make the correct number of connections with motoneurons (Sapir *et al.* 2004). In the absence of the transcription factor *Tlx3* (previously called *Rnx*), the interneurons that normally express *Tlx3* (dI3, dI5 and dILB) still seem to form and migrate as normal but the expression of some of the other transcription factors that are normally expressed by these cells is lost or not maintained (Qian *et al.* 2002). In a similar manner, embryos mutant for the transcription factor *Lmx1b* lose expression of some, but not all of the transcription factors normally expressed by dI5 and dILB cells, although in this case at least some of the affected cells also seem to have migration defects (Ding *et al.* 2004).

All of the above examples describe functions of individual transcription factors in specifying particular neuronal characteristics. However, it is also likely that transcription factors act in combination in at least some of these processes. The best understood example of a transcription factor that has different functions depending on its interactions with other transcription factors is *Lhx3*. *Lhx3* is expressed by both V2 interneurons and motoneurons. In V2 interneurons, *Lhx3* binds to a nuclear cofactor protein called *NLI*. However, in motoneurons the transcription factor *Isl1* displaces *Lhx3* from its binding domain on *NLI* and *Lhx3* binds to the C-terminal region of *Isl1* instead. These two different protein complexes have distinct functions, driving V2 and MN development, respectively (Thaler *et al.* 2002).

Taken together, all of these different results suggest that if we can determine the functions of different post-mitotic transcription factors, both singly and in combination, we may finally be able to define the genetic regulatory cascades that control specific neuronal characteristics such as migration pathways, axon morphologies, synaptic connectivity and neurotransmitter activity. However, this hypothesis is still largely untested as so far none of these transcription factors have been shown to directly regulate specific ion channels, receptors, neurotransmitters or axon guidance molecules.

4. CORRESPONDENCE BETWEEN MOLECULARLY DISTINCT NEURONS AND FUNCTIONALLY DISTINCT NEURONS

As this review has described, considerable progress has been made in determining how molecularly distinct populations of cells are specified at particular dorsal-ventral positions in the embryonic spinal cord. The current model suggests that the combination of post-mitotic transcription factors that a cell expresses will determine at least some of the resulting neuronal properties of that cell. If this hypothesis is correct then genetically determined/molecularly distinct cell populations in the embryonic spinal cord should correspond to physiologically and functionally identified classes of interneurons. Excitingly, these correlations are finally starting to be established.

The type of neuron that we know most about is motoneurons. Here, a clear correspondence has been

Figure 4. Spinal interneurons can be individually identified in zebrafish embryos and larvae. Taken from [Lewis & Eisen \(2003\)](#) with permission from Elsevier (Copyright, 2003). (a) Zebrafish embryonic spinal interneurons and two of the three major spinal axon tracts, the dorsolateral funiculus (DLF) and the ventrolateral funiculus (VLF). Redrawn from [Bernhardt *et al.* 1990, 1992](#)). Solid lines show ipsilateral axons (axons that extend on the same side of the embryo as the cell body). Dashed lines show contralateral axons (axons that cross the midline and extend on the opposite side of the embryo to the cell body). (b) Zebrafish larval spinal interneurons, axons of the paired Mauthner reticulospinal interneurons that descend into the spinal cord from the hindbrain and the third major spinal axon tract (MLF). Redrawn from [Hale *et al.* 2001](#)). In both cases, dorsal is up and anterior is left.

demonstrated between the combination of post-mitotic transcription factors that a cell expresses and the particular motoneuron subtype that it develops into. There are only a few different types of motoneurons and these can be easily identified in developing embryos as they extend their axons out of the spinal cord to distinct muscle territories. This has made it relatively easy to correlate cell morphology and gene expression and to investigate the functions of particular transcription factors in specifying motoneuron subtypes and specific neuronal characteristics (for reviews, see [Jessell 2000](#); [Shirasaki & Pfaff 2002](#)).

In contrast to motoneurons, there are many more classes of interneurons in the spinal cord and interneuron axons all remain within the spinal cord or brain where they are much harder to distinguish from one another. However, considerable progress has been made in recent years in connecting gene expression to cell morphology. Transgenic mice that express Green Fluorescent Protein (GFP) or the cell marker LacZ under the control of endogenous promoters have helped to identify the axonal projections and final cell body position of at least some of the different molecularly distinct interneuron populations in the mouse spinal cord ([table 1](#)). This enables us to correlate the axon trajectories and cell body positions of particular molecularly identified interneurons with descriptions of the morphologies and positions of different functionally identified interneurons. However, morphology alone does not necessarily predict physiology or function and researchers are only just starting to connect the molecular phenotypes of cells with physiological analyses. The only molecularly

identified interneurons that have been analysed in this way in the mammalian spinal cord are V1 interneurons. Electrophysiological analyses suggested that while V1 interneurons all have a similar morphology and at least most V1 interneurons make monosynaptic connections to motoneurons, V1 interneurons do not represent a single class of functional interneurons ([Wenner *et al.* 2000](#)). More recently, researchers used reporter constructs that are transported across synapses to confirm that V1 neurons form functional synapses with motoneurons. These experiments also identified Renshaw cells as a subset (about 10%) of V1 cells ([Sapir *et al.* 2004](#)). Renshaw cells are a particular type of functionally defined ventral interneuron that is involved in fine-tuning motor behaviours. Renshaw cells receive excitatory input from motor neurons and in turn inhibit motor neurons and other ventral interneurons ([figure 2b](#)). It is still not clear what types of interneurons the other 90% of V1 cells differentiate into, but it is likely that they include Ia and Ib inhibitory interneurons, which are two other types of ventral interneuron that inhibit motoneurons and hence contribute to the reflex pathways controlling locomotion ([Sapir *et al.* 2004](#) and see discussion below in §5a).

Examining the electrophysiology of cells and then correlating that with gene expression is a painstaking and technically very difficult process. Another way to connect molecularly distinct cells with interneurons with particular functions is to exploit the genetic differences between neurons to genetically ablate specific neuronal classes, by knocking out the functions of genes essential for their specification. In theory, this should enable us to assess which, if any, behaviours

Figure 5. The zebrafish spinal cord is smaller and simpler than the spinal cord in birds and mammals. (a) Lateral views (on the left) and cross-sections (on the right) showing RNA expression of *eng1b* and *vsx1* in the zebrafish spinal cord at about 20 h of development. In all cases dorsal is up and anterior is left. Red dotted lines demarcate the edge of the spinal cord. The names of homologous mammalian genes are given in parentheses. (b) A schematic cross-section of a vertebrate spinal cord. Engrailed-expressing interneurons are represented in red and Chx10/Vsx1-expressing interneurons are represented in green. The purple rectangle indicates the region shown in the zebrafish cross-sections in (a); the pale blue rectangle indicates the part of the spinal cord shown in (c). (c) Taken from Briscoe *et al.* (2000, fig. 5) with permission from Elsevier (Copyright, 2000). A cross-section of the ventral right-hand corner of the spinal cord showing En1 and Chx10 expression in a chick embryo at HH stage 20–24. Note that there are many more cells expressing each of these genes in the chick cross-section than there are in the zebrafish cross-sections. In most cases, in cross-sections of the zebrafish spinal cord a maximum of one cell on each side of the spinal cord expresses a particular transcription factor.

those neurons are required for and to directly connect individual genes with particular neuronal functions. One potential problem with this approach is that in many cases, these mutant embryos may not survive long enough or may have too many other general developmental problems for us to analyse the effects of the loss of the neurons on specific behaviours (e.g. Pierani *et al.* 2001). However, recent experiments have shown that it is possible to use an *in vitro* spinal cord preparation from embryonic or early postnatal mice to examine locomotor (movement) behaviours even in embryos that normally die at birth (Lanuza *et al.* 2004). The locomotion observed in these preparations is called ‘fictive locomotion’: it is induced *in vitro* using chemicals and observed by electrophysiological recordings from motor nerves. These techniques were used to show that either $V0_D$ neurons, or $V0_V$ and $V0_D$ neurons, control the alternating left–right activity of the motor neurons that innervate hindlimb muscles and hence are essential for correct walking movements (Lanuza *et al.* 2004). Both $V0_V$ and $V0_D$ neurons express *Dbx1* as progenitors and both classes of neuron

Figure 6. The tadpole and its spinal neurons. Taken from (Li *et al.* 2004) with permission from the Society for Neuroscience (Copyright, 2004). The *Xenopus* tadpole at the time of hatching (stage 37/38), with its head to the left, showing the eye, the border between midbrain and hindbrain (m/h), and the spinal cord lying under segmented swimming muscles. Below is a schematic of a length of spinal cord viewed from the side to show the neurons. Glycinergic aINs (purple) have ascending and descending axons that contact and inhibit dorsolateral commissural INs (dlc), dorsolateral ascending interneurons (dla) and motoneurons. dlc and dla interneurons are excitatory sensory pathway interneurons, excited by skin sensory, touch-sensitive Rohon-Beard sensory neurons (RB) that have no dendrites. aINs may also inhibit excitatory dINs, glycinergic reciprocal inhibitory cINs, and other aINs that are active during swimming. For each neuron class, the circle/oval is the soma; oblique lines indicate the dorsal–ventral extent of the dendrites; and the thin line shows the axon projection (asterisk indicates that the axon crosses ventrally to the opposite side of the spinal cord). Each class of neuron forms a longitudinal column of 50–150 neurons on each side of the cord. dIN neurons are probably equivalent to VeLD and/or CiD neurons in zebrafish; cIN neurons are probably equivalent to CoSAs and/or CoBs; aIN interneurons are probably equivalent to CiAs; dla interneurons are probably equivalent to DoLAs and dlc interneurons are probably equivalent to CoPAs.

have similar migration patterns and axons that cross the midline and contact motoneurons on the opposite side of the spinal cord (Pierani *et al.* 2001; Lanuza *et al.* 2004). During fictive locomotion, mouse embryos mutant for *Evx1* (that lack $V0_V$ neurons) have normal alternating left–right motor control but mouse embryos mutant for *Dbx1* (that lack $V0_V$ and $V0_D$ neurons) have an increased incidence of cobursting between left and right flexor/extensor motor neurons (Lanuza *et al.* 2004). This is consistent with earlier studies that showed that *Evx1* mutant mice, which unlike *Dbx1* mutant mice are viable, have no major defects in movement (Moran-Rivard *et al.* 2001). Taken together, these results suggest that either $V0_D$ neurons are specifically required for walking movements or that $V0_V$ and $V0_D$ neurons are similar enough to each other that loss of just one population does not produce a behavioural phenotype. To distinguish between these possibilities it will be necessary to specifically remove

V0_D but not V0_V neurons and observe whether this affects locomotor control.

The genetic differences between neurons can also be exploited to alter specific neuronal populations, by removing genes that are required for only some aspects of neuronal specification. These studies can also be very useful for correlating genetically distinct neuronal populations with specific neuronal functions. For example, embryos mutant for either *Lmx1b* or *Tlx3* lose or do not maintain *Prrxl1* (previously called *Drg11*) and *Ebf3* expression in the dorsal horn and *trkA*+ sensory neurons no longer enter the dorsal horn (Qian *et al.* 2002; Ding *et al.* 2004). This is a similar, but more severe phenotype than that observed in *Prrxl1* mutants, where the growth of sensory neurons into the dorsal horn is delayed and biased towards the medial and away from the lateral regions (Chen *et al.* 2001b). *Prrxl1* is also required for the differentiation and survival of a particular class of nociceptor sensory neurons in the dorsal horn, and mice that lack *Prrxl1* have normal sensorimotor and locomotor function but their sensitivity to painful stimuli is reduced (Chen *et al.* 2001b). All of these examples strongly suggest that the interneurons that normally express *Lmx1b* and *Tlx3* and migrate to the superficial laminae of the dorsal horn (probably DIL_B neurons) are involved in attracting *trkA*+ sensory neurons into the dorsal horn and receiving and integrating pain signals.

5. USING ZEBRAFISH AS A MODEL FOR SPINAL CORD DEVELOPMENT

As discussed in §4, several recent developments have started to link cells that express particular transcription factors in the embryonic mammalian spinal cord with interneurons with particular morphologies and occasionally even particular functions. These discoveries have significantly increased our understanding of how the nervous system develops and functions but we are still a long way from a complete understanding of these processes. Progress in understanding mammalian spinal cord development is slowed down by the complexity of the mammalian nervous system and by the time and cost involved in inactivating specific genes in mice. In this light, recent research that is starting to examine how different interneurons develop and function in zebrafish is very interesting as the results suggest that we can use the simpler nervous system of the zebrafish to learn many of the basic principles of how the vertebrate spinal cord is organized and functions.

The zebrafish spinal cord only has a small number of distinct types of interneurons, all of which can be uniquely identified based on their cell morphology (cell body shape, size and position and axonal trajectory; Bernhardt *et al.* 1990; Hale *et al.* 2001; figure 4 and compare to Jankowska 2001). We also know which neurotransmitters most of these morphologically identified zebrafish interneurons express, allowing us to hypothesize what their functions may be (Bernhardt *et al.* 1992; Martin *et al.* 1998; Higashijima *et al.* 2004a–c; McLean & Fetcho 2004). In addition, several researchers are actively investigating the functions of specific morphologically identified zebrafish inter-

neurons in particular behaviours (Ritter *et al.* 2001; Drapeau *et al.* 2002; Higashijima *et al.* 2003, 2004b and references therein) and/or are starting to establish which transcription factors these morphologically identified interneurons express (Tamme *et al.* 2002; Higashijima *et al.* 2004b; K. E. Lewis, unpublished data).

All the data so far suggests that the mechanisms of spinal cord patterning are highly conserved between zebrafish and mammals. As in birds and mammals, Hh signals specify ventral spinal cord fates in zebrafish (Beattie *et al.* 1997; Chen *et al.* 2001a; Lewis & Eisen 2001; Varga *et al.* 2001) and BMP signals probably specify dorsal interneurons (Barth *et al.* 1999; Nguyen *et al.* 2000). Many (maybe all) of the transcription factors that are expressed in the spinal cord of birds and mammals are also expressed in the zebrafish spinal cord and in the vast majority of cases these genes are expressed in similar dorsal–ventral positions (Lewis & Eisen 2003; K. E. Lewis, unpublished data; figure 5). The major difference is that the domains of expression are much smaller in zebrafish embryos than in birds or mammals, correlating with the much smaller size of the zebrafish spinal cord (figure 5). This enables us to analyse specification of zebrafish neurons at the level of single cells as well as populations of cells.

Zebrafish are also a powerful system for combining genetic and functional studies. Zebrafish embryos develop outside the mother and hence are readily accessible. They are also optically transparent, allowing us to follow the development of individual cells in live embryos, analyse the electrical activity of individually identified neurons and easily correlate gene expression with neuronal morphology and function. Several behavioural mutants have been identified in zebrafish (e.g. Westerfield *et al.* 1990; Granato *et al.* 1996; Fetcho & Liu 1998 and references therein) and additional mutants can easily be screened for. Several existing mutations have already been analysed in detail and in some the molecular lesion has been identified (Ribera & Nüsslein-Volhard 1998; Sepich *et al.* 1998; Lorent *et al.* 2001; Ono *et al.* 2001, 2002; Behra *et al.* 2002; Cui *et al.* 2004, 2005; Hirata *et al.* 2004, 2005). In addition, analysing the roles of specific genes by loss-of-function and gain-of-function experiments is technically simple in zebrafish and much faster than making specific mouse mutants. Modified oligonucleotides called morpholinos can be injected into embryos to ‘knock-down’ specific gene functions and RNA can be injected to ectopically activate particular genes. Consequently, zebrafish can provide a potential bridge between the extensive studies on neuronal morphology and function that have been done in the simple systems of the lamprey and the frog (Fetcho 1992; Roberts 2000) and the more molecular and genetic analysis of spinal cord development that has been conducted in birds and mammals. For example, the different classes of interneurons involved in coordinating movements have been identified and extensively studied in the *Xenopus* tadpole (Roberts 2000 and references therein; Li *et al.* 2002, 2004; figure 6). While the same level of functional analysis has not yet been conducted in zebrafish, there is a close correspondence between the morphologies of the different classes of interneurons

identified in zebrafish and *Xenopus* embryos (Roberts 2000) and the evidence so far suggests that these interneuron classes are also functionally homologous (Higashijima *et al.* 2004b; Li *et al.* 2004). However, the zebrafish is a much better genetic organism than *Xenopus* and in particular the ease of making transgenic lines makes it a better organism for correlating gene expression and morphology and investigating the functions of different transcription factors.

Recently, techniques have been developed that allow researchers to analyse the electrical activity of individually identified neurons during specific behaviours in live zebrafish embryos. Several distinct motor behaviours have been identified, each of which starts to occur at a precise time in development (Saint-Amant & Drapeau 1998). In addition to standard electrophysiological techniques (Drapeau *et al.* 1999), calcium indicator dyes can be used to detect whether individually identified neurons fire action potentials during specific behaviours (Fetcho & Liu 1998; Fetcho *et al.* 1998; Higashijima *et al.* 2003). The latter technique can be performed non-invasively and it is technically simpler than making electrophysiological recordings. The calcium dyes are fluorescent and even a single action potential elicits a measurable fluorescence increase within the cell body of a labelled neuron. These reagents can be ectopically expressed under the control of particular gene promoters, or by random mosaic over-expression or by retrograde labelling (Fetcho & Liu 1998). Using a confocal microscope, individual labelled cells can then be identified unambiguously by their morphology in partially immobilized zebrafish embryos and their activity monitored while particular behaviours like swimming or an escape response are elicited in the embryo. These techniques enable functional analysis of different zebrafish neurons in wild-type embryos, mutant embryos, and experimentally manipulated embryos.

(a) Zebrafish CiA interneurons and mammalian V1 interneurons are genetically and functionally homologous

Excitingly, a recent paper has shown that the cells that express Engrailed 1b in the zebrafish spinal cord constitute a multi-functional class of interneurons that share many characteristics with V1 interneurons in birds and mammals (Higashijima *et al.* 2004b). In the zebrafish spinal cord, Engrailed 1b is expressed exclusively by a class of ipsilateral ascending inhibitory interneurons called circumferential ascending (CiA) neurons that are rhythmically active during swimming (Higashijima *et al.* 2004b). Like V1 neurons, CiA neurons inhibit motoneurons and other ventral interneurons (Higashijima *et al.* 2004b; Sapir *et al.* 2004). CiA neurons also inhibit dorsal sensory neurons involved in sensory gating, suggesting that a subset of V1 neurons may also have this function. In contrast to V1 neurons, individual CiA neurons are multi-functional, suggesting that CiA neurons may be a more primitive cell type that has given rise to several more specialized subpopulations of ipsilateral ascending inhibitory interneurons in birds and mammals (Higashijima *et al.* 2004b). This suggests that studies of the zebrafish spinal cord may reveal a primitive

organization, where particular transcription factors specify a basic set of different functional classes of interneurons, many of which have probably further specialized into related, but distinct interneuron subclasses during the evolution of birds and mammals. If this is true, not only does the zebrafish spinal cord provide a simple system for analysing interneuron specification and function, and an important system for investigating how the spinal cord has evolved in vertebrates, but our findings from studying zebrafish interneuron development may predict both the functions of particular genetically specified mammalian interneurons and the roles of specific transcription factors in determining neuronal characteristics in mammals. For example, the ipsilateral inhibitory interneurons involved in sensory gating have not yet been identified in birds and mammals. If the multi-functional zebrafish CiA neurons have really evolved into different subclasses of En1 expressing interneurons in mammals, then we would expect that a subset of V1 interneurons would be these as-yet-undiscovered sensory gating interneurons.

Interestingly, even though Engrailed expression correlates with the specification of remarkably similar neuronal populations in zebrafish, mouse and probably *Xenopus* (Higashijima *et al.* 2004b; Li *et al.* 2004; Sapir *et al.* 2004), as discussed above in §3d, En1 is not required for most of the characteristics of V1 neurons. In mouse embryos mutant for *En1*, the early development of V1 interneurons appears to be completely normal. The only phenotypes discovered so far are that V1 axons have defects in pathfinding and fasciculation within the VLF (Saueressig *et al.* 1999) and Renshaw cells no longer make the correct number of connections with motoneurons (Sapir *et al.* 2004). This suggests that there may be other transcription factors that are expressed in common between V0 and CiA interneurons that specify more general characteristics of these neurons.

6. LOOKING TO THE FUTURE

To develop a comprehensive understanding of vertebrate spinal cord development and function we need to conduct experiments in a variety of model organisms, partly because different animals have different strengths and weaknesses for this research. In addition, studying different vertebrates, and in particular those that have a very distant common ancestor, should help us to distinguish fundamental properties of the vertebrate spinal cord that have been conserved throughout evolution, newer features that have evolved in particular vertebrate lineages and species specific phenomena. This will also help us to understand how the particularly complex mammalian spinal cord has evolved.

A number of exciting techniques are now available that should dramatically facilitate future investigations into spinal cord development. For example, many interneurons migrate during their development, but how these cell movements are regulated is poorly understood. We can now use transgenic zebrafish embryos with fluorescently labelled neurons to observe cell movements in live embryos and investigate how

these cell movements are controlled. We can also start to determine how neurons connect up with each other, using recently developed reporter constructs that are transported across active synapses (Coen *et al.* 1997; Yoshihara *et al.* 1999; Miana-Mena *et al.* 2002; Sapir *et al.* 2004). Transgenic lines that express these constructs in particular subsets of spinal cord interneurons can be used to map the neural circuits that these neurons are involved in. In zebrafish embryos, these constructs can also be expressed in specific cells by activating heatshock constructs or caged RNA with a laser. As we identify molecular markers for functional subtypes of interneurons this will also enable us to ablate or manipulate those neurons without affecting the development of the rest of the animal. For example, in mice Cre-Lox technology could be used to specifically knock-down genes required for the survival or correct differentiation of particular neurons, only in the neurons concerned. In mice or zebrafish, neurons could be ablated at particular stages of development or even in adults by immunotoxins. For example, human interleukin-2 receptor α subunit could be expressed under the control of a promoter specific to particular neurons and animals treated with an immunotoxin consisting of a monoclonal antibody to this protein fused to a truncated form of *Pseudomonas* exotoxin (Kobayashi *et al.* 1995). In a similar manner, a temperature-sensitive allele of *shibire shi* (*ts1*) could be used for conditional and reversible inactivation of specific neurons. Shibire protein is essential for synaptic vesicle recycling, and *Drosophila shi* (*ts1*) is semi-dominant, a simple temperature shift leads to fast and reversible effects on synaptic transmission. For example, in *Drosophila shi* (*ts1*) expression in cholinergic neurons creates a temperature-induced, reversible paralysis and *shi* (*ts1*) expression in photoreceptor cells produces temperature-dependent reversible blindness (Kitamoto 2001). In zebrafish, specific interneurons can also be photo-ablated using a laser if they can be identified in live embryos by their morphology or GFP expression (e.g. Fetcho & Liu 1998; Liu & Fetcho 1999). In both mice and zebrafish gene-specific promoters could be used to express constitutive active or inactive receptors or particular ion channels in specific neurons and hence alter their activity. This sort of analysis has already been exploited in *C. elegans* where a dominant glutamate receptor mutation was expressed in specific interneurons that control locomotion (Zheng *et al.* 1999). All of these very powerful techniques will enable us to remove the functions of individual classes of neurons, or even (for example, by combining transgenic lines) combinations of neuronal classes at different times in development, and observe the effects on behaviour. In this way, we can now start to study the functions of particular neurons, whether these functions change over time and whether any compensation or plasticity mechanisms exist within the spinal cord.

Most general over-expression techniques can be used to some extent in many animals, but transgenic lines can only be easily made in mice and zebrafish. Transgenic methods are not trivial in either animal but they are considerably cheaper and faster to use in zebrafish. A particularly powerful strategy for

increasing our understanding of spinal cord development and function might therefore be to use these different techniques to analyse all of the steps of spinal cord development in zebrafish embryos: the specification of different early populations of cells, their cell movements and development of specific morphologies and axon trajectories, their connections with other cells and formation of neural circuits and their electrical activities and functions in specific behaviours. All of these experiments are now technically feasible and this research would provide us with a comprehensive description of how genes regulate at least some behaviours in a simple vertebrate spinal cord. This description could then be built upon by studies examining the differences in the mammalian spinal cord. In this way, I anticipate that we could achieve major advances in this field over the next few years.

I thank Roger Keynes, Charles Kimmel and Alan Roberts for comments on a previous version of this manuscript. I apologize to any colleagues whose work I have failed to cite. I am supported by a Royal Society University Research Fellowship and work in my lab is funded by both the Royal Society and the Cambridge Isaac Newton Trust.

ENDNOTE

¹I use the term 'cardinal' to denote a major class of neurons that shares many fundamental characteristics. In mammals, at least some of these cardinal classes of neurons develop into several related but specialized sub-populations (e.g. see discussion in §2b and §5a).

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