CHAPTER

4

Determination and Differentiation

The nervous system is a coral reef of the body where evolution and development have collaborated to produce an extraordinary diversity of cell types. Neurons show enormous variety in cellular anatomy, physiological function, neurochemistry, and connectivity. For example, granule cells of the cerebellum are tiny, and have simple dendrites and bifurcated axons that release the excitatory transmitter glutamate, whereas cerebellar Purkinje cells are huge, have an impressively complex and electrically active dendritic tree, a single long spiking axon, and release the inhibitory neurotransmitter GABA. The differences between neurons can be much more subtle. All the motor neurons of the spinal cord share a common morphology, chemistry, physiology, and circuitry, yet they are distinctly specified molecularly so that they connect with particular presynaptic partners and postsynaptic muscles.

The fates of some neurons, particularly those of invertebrates, are the products of particular lineages. The fates of others, particularly those in vertebrates, appear to depend more on the local environment. Sydney Brenner suggested that neurons are either European or American. A neuron is European if its fate is largely the result of who its parents were. For American neurons, it is more about the neighborhood where they grew up. When one looks closely, however, it turns out that fate is not strictly controlled by either lineage or environment alone. Usually, it is the mixture of the two that is essential; the adoption of a particular fate is a multistep sequential process that involves both intrinsic and extrinsic influences. A progenitor cell may be externally influenced to take a step along a particular fate pathway, and so the unborn daughter of that cell has also, in a sense, taken the same step. A signal from the environment may act upon this daughter cell to refine its fate further, and the response of the daughter cell to the signal is to express an intrinsic factor consistent with its limited fates.

The environment in which neural progenitors divide and give rise to neurons is rich with diffusible molecules, cell surface proteins, and extracellular matrix factors. These extrinsic signals influence the genes that developing neurons express, which direct neuronal shape, axonal pathways, connectivity, and chemistry. The number of genes used to carry out this task of specification throughout the nervous system is impressive. It has been estimated that half of an organism's genes are expressed exclusively in the nervous system. Most of these are involved in various aspects of neuronal differentiation.

Some of the basic techniques that are used in approaching these questions are shown in Figure 4.1. Transplantation is a good technique for finding out whether a cell's fate has been intrinsically specified. For example, a progenitor from a donor animal is transplanted to a different part of a host animal. If the fate of the cell is unaltered by putting it in this new environment, then the cell is "autonomously determined" at the time of transplantation. If, however, the cell adopts a new fate, consistent with the position to which it was transplanted, then the fate at the time of transplantation is still flexible and can be "determined nonautonomously." Putting cells into tissue culture is another valuable technique. By isolating a cell from the embryo entirely, it is possible to assay the state of determination of a cell in the absence of all interactions. An advantage of this experimental system is that the culture medium and substrate can be controlled. In this way, potential extrinsic cues can be added and assayed for their effect on fate choice.

A very informative approach for studying the processes that lead neurons down particular differentiation pathways, at least in terms of identifying the factors

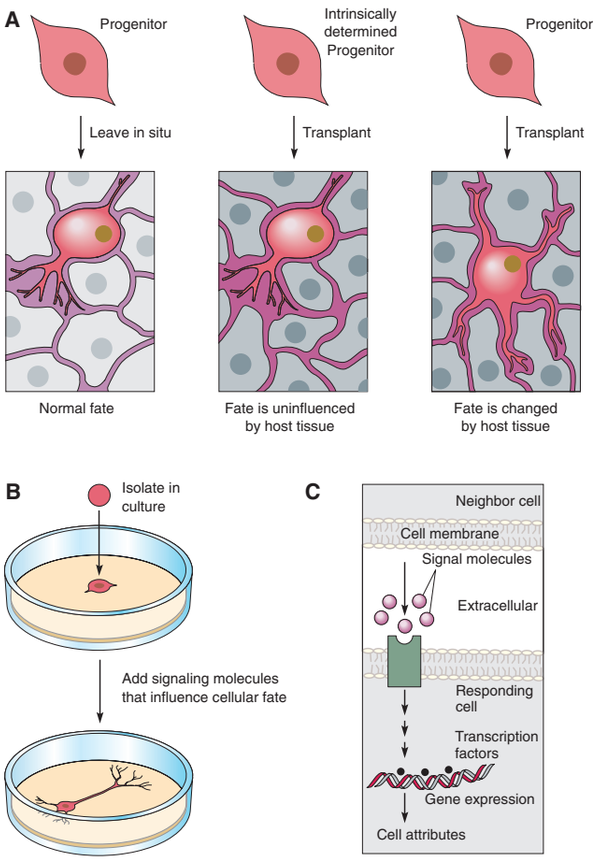

FIGURE 4.1 A. Testing fate by transplantation. On the left, a neural progenitor left in its normal environment turns into a particular type of neuron. In the middle, an intrinsically determined progenitor's fate is unchanged by transplantation to a different environment. On the right is an example of a progenitor whose fate is determined extrinsically and so is changed by transplantation to a different environment. B. An undifferentiated progenitor is placed into a culture dish, and signaling molecules are tested, which may influence the fate that the cell takes as it differentiates into a neuron. C. An extracellular signal originating from one cell can influence the fate of nearby cells by causing the responding cell to change its gene expression pattern.

that influence determination, is genetic manipulations such as mutational and transgenic analyses. Mutations in particular genes can alter the fate of certain types of neurons. With a genetic approach it is possible not only to show where and when the normal fate decisions are made but also to identify the gene product in question. Forward genetics uses random mutagenesis to define new genes that have effects on neural differentiation, while reverse genetics uses molecular engineering to knock out or overexpress particular genes (see Chapter 2) that are candidates for roles in neuronal fate determination or differentiation. Genetics combined with transplantation or culture can reveal whether neuronal phenotypes are extrinsically regulated by the gene in question, as in the case of a gene that codes for a secreted factor, or intrinsically regulated, as in the case of a gene that codes for the receptor to such a factor.

This chapter examines the several facets of cell fate determination and differentiation, which have been investigated using such techniques. Each aspect is brought to light in a different system, and it is only through looking at several systems that one can begin to appreciate the full range of cellular and molecular mechanisms that lead a set of relatively simple looking progenitor cells to take on thousands of different neuronal fates.

TRANSCRIPTIONAL HIERARCHIES IN INVARIANT LINEAGES

As we discussed in Chapter 1, time-lapse studies of the development of the nervous system of the nematode *C. elegans* show that every neuron arises from an almost invariant lineage (Figure 4.2). In this system, the progenitors are uniquely identifiable by their position and characteristic patterns of division. Ablation of one of these progenitors usually leads to the loss of all the neurons in the adult animal that arise from that progenitor, indicating neighboring cells cannot fill in the missing fates. This is called mosaic development. To understand how these different precursors generate specific neurons, a genetic approach has been used, and mutants have been found that interfere with the development of particular neurons. These mutants are then used to dissect the mechanisms of neuronal fate. In this system, acquisition of neural identity is largely the result of a multistep, lineage-dependent, process of determination.

One of the best examples of such an analysis is that of the specialized mechanosensory cells in nematodes studied by Martin Chalfie and his colleagues (Chalfie and Sulston, 1981; Chalfie and Au, 1989; Chalfie, 1993; Ernstrom and Chalfie, 2002). Most nematodes wiggle forward when touched lightly on the rear and backward when touched on the front. By prodding mutagenized nematodes with an eyelash hair attached to the end of a stick, Chalfie and colleagues were able to find mutants that had lost the ability to respond to touch. Many touch insensitive worms have mutations in a group of genes involved in the specification of the mechanosensory cells. Mutations in the gene *unc-86* result in the failure of the mechanosensory neurons to form. *Unc-86* encodes a transcription factor that is expressed transiently in many neural precursors and particularly in the lineage

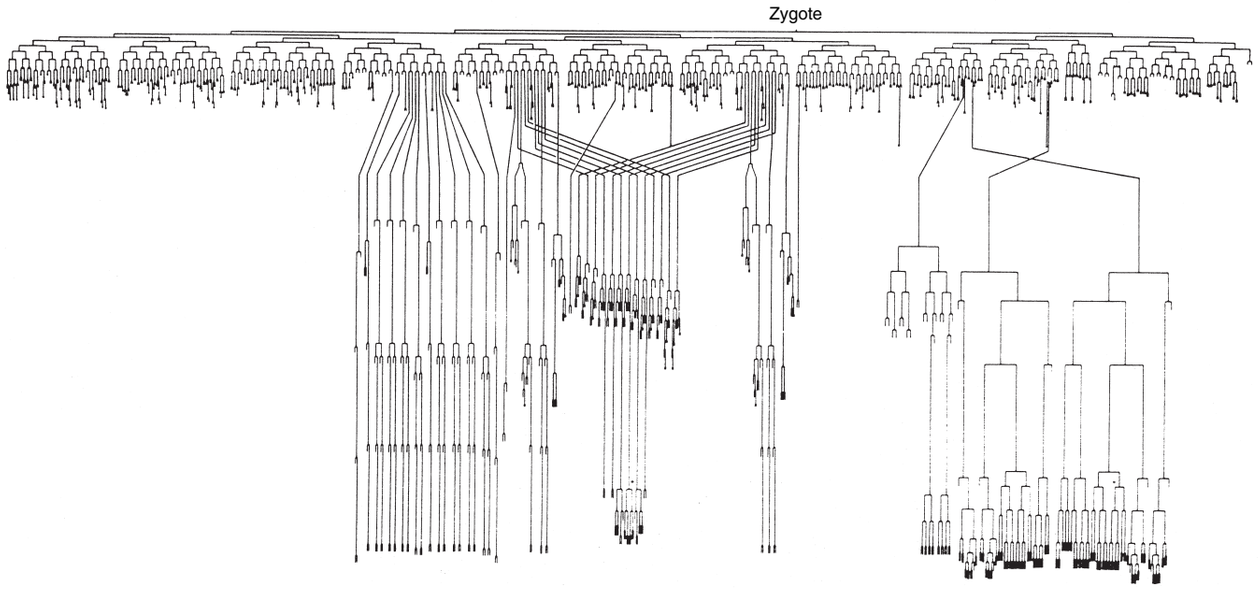

FIGURE 4.2 Complete lineage of *C. elegans* hermaphrodite. (Based on Sulston et al., 1983)

produced by a cell called Q. In wild-type animals, Q divides into two daughter cells, Ql.a and Ql.p (Figure 4.3A). Both of these cells divide once more, but only Ql.p produces a touch cell. The *unc-86* gene is turned on only in the Ql.p. and a mutation in *unc-86* (Figure 4.3B) results in the "transformation" of Ql.p into a cell that behaves like its mother, Q. We call this cell Q′. This transformed cell continues to divide, producing Q1.a′ and Q1.p′, but in the continued absence of *unc-86* function, the Q1.p′ transforms into Q″, which continues to behave like its mother Q′ and its grandmother Q. Thus, mutations in *unc-86* affect the lineage of touch cells; mechanosensory neurons are never born in these mutants.

Another gene uncovered in Chalfie's screen of touch mutants is named *mec-3.* In these mutants (Figure 4.3C), the cells that would be touch sensitive are born, but they do not differentiate into mechanosensory neurons. Instead, they turn into interneurons. Thus, the *mec-3* mutation affects neural subtype determination. The *mec-3* gene codes for a transcription factor that is a member of the LIM-homeodomain family. Interestingly, the transcription of the *mec-3* transcription factor is directly regulated by the *unc-86* transcription factor. Cells fated to become touch cells all express *unc-86* at first, and this transcription factor binds to the regulatory sequence of DNA that controls the transcription of the *mec-3* gene. Thus, *unc-86* mutants do not express *mec-3.* However, in normal animals, the protein UNC-86 leads to the expression of MEC-3, and when these two proteins are expressed in the same cell (the cell that will become a mechanosensory neuron), they physically interact to make a heterodimeric transcription factor with new specificity that activates genes that neither MEC-3 nor UNC-86 can activate on their own. Several of these are defined by mutations in other genes that cause touch insensitivity, such as the *mec-7, mec-12,* and *mec-17* genes. These three genes encode proteins that are used in the differentiation of the specialized touch cell cytoskeleton (Figure 4.3D). This system provides an excellent example of a simple hierarchical cascade of transcription factors, one regulating and interacting with the next, the end result of which is to turn on genes that the cell uses to realize its fate. Using a genomic approach, Chalfie and colleagues (Zhang et al., 2002) have tried to find even more genes involved in the touch cell pathway by looking for differences in profiles of all expressed genes in normal animals versus *mec-3* mutants. This approach identified up to 50 more genes in the pathway downstream

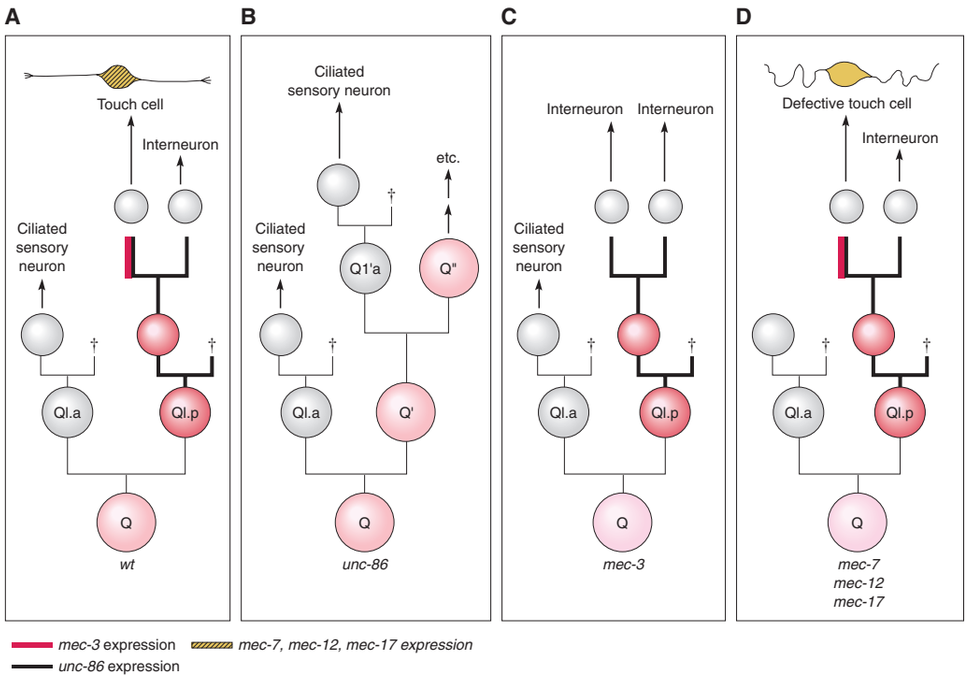

FIGURE 4.3 Intrinsic determinants at different steps on a neural lineage in the nematode. A. Normal lineage. B. If Q1.p cannot express *unc-86*, it becomes Q', a copy of its mother, Q. The result is a repeat of the previous division, which results only in Q progenitors and ciliated sensory neurons. C. *Mec-3* needs to be expressed in the touch cell. If it is not, as in *mec-3* mutants, this cell turns into an interneuron like its sister. D. In *mec-7*, *mec-12*, and *mec-17* mutants, the touch neuron is correctly specified but differentiates missing critical components of its morphology or function.

of *mec-3*, genes that are likely important for the function of the mechanosensory neurons.

Another set of *C. elegans* neurons that have been studied in detail are the hermaphrodite-specific egg-laying neurons (Desai et al., 1988; Desai and Horvitz, 1989). Mutants in genes involved in the determination or function of these neurons are unable to lay eggs. The result is that the self-fertilized eggs hatch inside the mother and begin to feed within their mother's uterus. The larvae proceed to devour their mother from the inside. Eventually, with only her epidermis intact, she becomes a bag of wriggling larval worms that in their hunger eventually eat through her cuticle into the world. Mutant lines in these genes thus seem to give their offspring a protected start in life characteristic of viviparous species but at what appears to be a mother's ultimate altruistic sacrifice. The "bag of worms" mutants have a phenotype that is easy to detect, and so a large collection of such mutants has been identified. Twenty or so genes have been found to define hierarchical transcriptional cascades affecting egg-laying neuron development. Surprisingly, only one of these is also necessary for the proper development of the touch cells. This is our friend *unc-86*. The role of *unc-86* in the determination of the egg-laying neurons, however, is quite different. Instead of controlling lineage as it does in mechanosensory cells, it regulates neurotransmitter expression and axon outgrowth in the egg-laying neurons.

The fact that there is surprisingly little overlap in the genes that are involved in these two systems suggests that the molecular cascades of neuronal determination must be complex and highly individualized. However, there are similarities that are worth emphasizing. In the case of both the egg-laying and the mechanosensory neurons, there is a hierarchical pathway, rich in transcription factors that operate through the specific lineages. These factors regulate other intrinsic transcription factors in a molecular cascade whereby the lineage, the specification, the differentiation, and finally the physiological properties of the neurons are established through a series of successive stages. This molecular strategy, we will see, is also used in the determination of neurons in most other species.

SPATIAL AND TEMPORAL COORDINATES OF DETERMINATION

The CNS of an insect develops from a set of individual neuroblasts that enlarge within the epithelium of the neurogenic region of the blastoderm and then delaminate to the inside, forming a neuroblast layer. All these neuroblasts, we learned in Chapter 1, express proneural transcription factors of the *Achaete-Scute* family that give them a common "neural" specification. However, each neuroblast is an individual and through successive divisions reproducibly gives rise to a unique set of neurons (Figure 4.4). How do these neuroblasts get their specific identities? They are arranged in reproducible columns and rows and so can be identified by their position. In Chapter 2, we discussed how the *Drosophila* embryo is finely subdivided in the anterior to posterior axis into stripes of expression of particular combinations of gap genes, pair-rule genes, *Hox* genes, and segment polarity genes. These genes provide neuroblasts with intrinsic positional information that reflects their location along the antero-posterior axis (Figure 4.5). *Hox* genes are expressed in the middle and posterior portions of the neural primordium, and the "head gap" genes are expressed more anteriorly in nested domains and provide positional information to the neuroblasts that give rise to particular brain regions or segments. Segment polarity genes control positional information within each individual segment (Bhat, 1999). These anterior-posterior (AP) positional identity genes play important roles role in determining the identity of the neuroblasts as illustrated by the loss and/or duplications of particular sets of neurons. For example, *Wingless (wnt)* and *Hedgehog* proteins activate the expression of a gene called *huckebein* in some neuroblasts, and the transcription factors *Engrailed* and *Gooseberry* repress *huckebein* expression in other neuroblasts, thus establishing the precise pattern of huckebein protein in specific neuroblast lineages (McDonald and Doe, 1997).

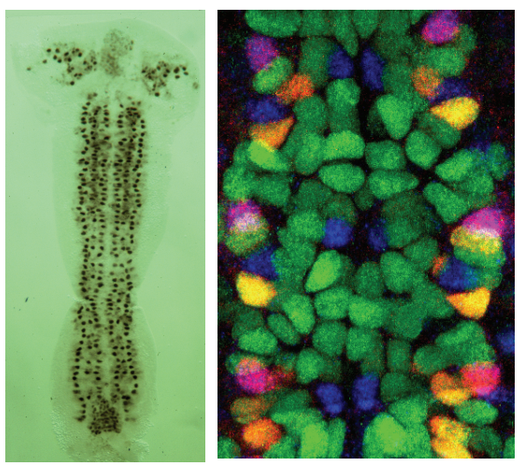

FIGURE 4.4 Neuroblasts of the *Drosophila* embryo. A. Shows the rows of neuroblasts labeled with an antibody to a late neuroblast-specific protein called Snail. B. Shows neuroblasts labeled with three different antibodies to the different neuroblast-specific proteins Hunchback, Eagle, and Castor. (Photos courtesy of Skeath and Doe)

Another set of genes divides the embryo and the nervous system along the dorsoventral axis. Three *homeobox* genes, *vnd*, *ind*, and *msh*, are expressed in longitudinal stripes within the neural ectoderm (Cornell and Ohlen, 2000). *vnd* is expressed in neuroblasts closest to the ventral midline, *msh* is expressed in the most dorsolateral stripe of the neurectoderm, and *ind* is expressed in an intermediate stripe between these two (Figure 4.5C). As is the case for the AP genes, mutations in these genes lead to loss of the neuroblast fates that normally express the mutated gene. The mechanism responsible for setting up these stripes involves responses of the promoters of these genes to threshold levels of the morphogen Dpp, which forms a gradient of expression from dorsal (high) to ventral (low). Once set up, the boundaries between the stripes of *vnd, ind,* and *msh* are sharpened by mutual repression.

A neuroblast in any position can thus be uniquely identified by expression of these spatial coordinate markers of latitude and longitude (Figure 4.5). These genes specifying position information along these two Cartesian axes collaborate to specify a positional identity for each central neuroblast in the developing organism. Once expressed in a neuroblast, the spatial coordinate genes are inherited by all the progeny of these cells, and act as intrinsic determinants of neuronal fate.

Each neuroblast divides asymmetrically to produce a copy of itself and a ganglion mother cell (GMC). The neuroblast divides several more times giving rise in ordered succession to a set of GMCs (see Chapter 1). Each GMC can thus be identified not only by the position of the neuroblast from which it arises but also from the order of its generation (e.g. whether it is the first, second, or third GMC to arise from a particular neuroblast). The first GMCs of a neuroblast lineage tend to lie deeper in the CNS and generate neurons with long axons, whereas the later arising GMCs stay closer to the edge of the CNS and generate neurons with short axons. In generating GMCs, neuroblasts go through a temporally conserved program of transcription factor expression (Figure 4.5 D and E). In the stages when the first GMCs are generated, most neuroblasts

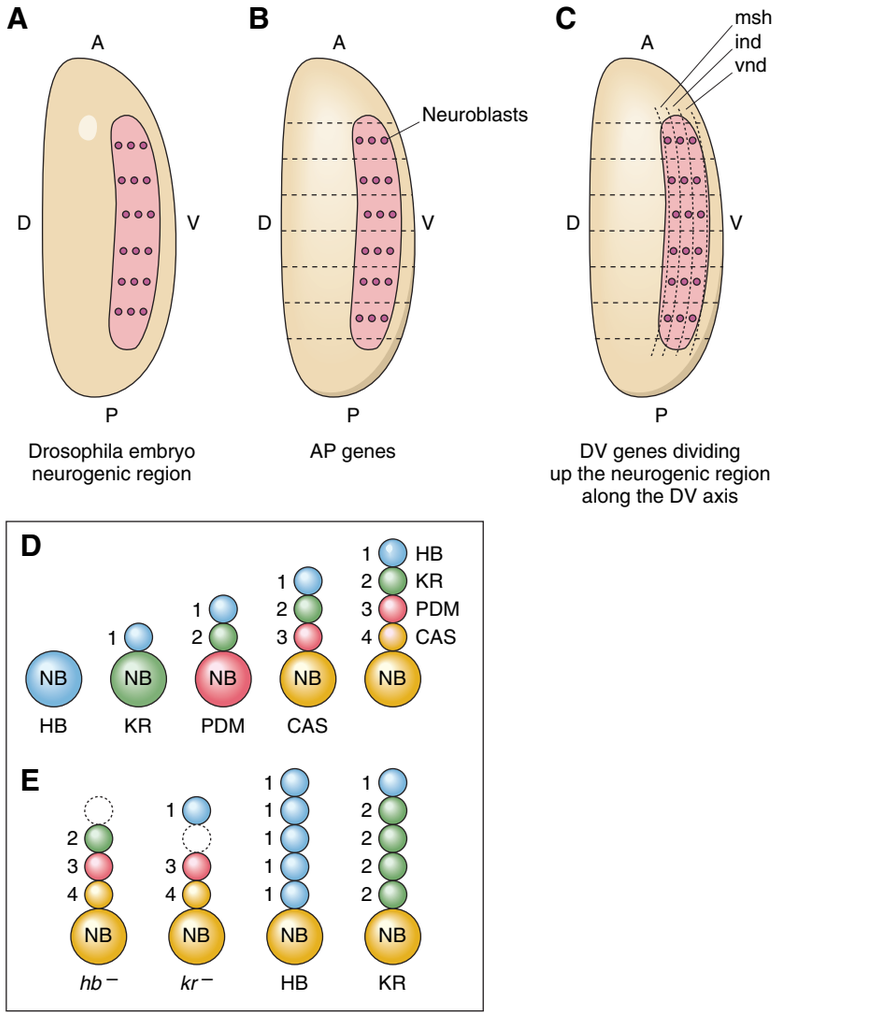

FIGURE 4.5 Positional and temporal coordinates of neuroblasts in *Drosophila*. A. The neurogenic region of a *Drosophila* embryo showing the rows and columns of neuroblasts. B. The embryo and the neurogenic region is divided segmentally into AP domains as described in Chapter 2. C. The neurogenic region is further divided into DV domains by the expression of transcription factors such as msh, ind, and vnd, creating a grid whereby each neuroblast has its own specific spatial coordinates. D. Neuroblasts transiently express temporally coordinated transcription factors *Hb,* then *Kr,* then *Pdm,* then *Cas,* but their progeny the GMCs maintain the transcription factor profile that was present at their birth. E. In *Hb* and *Kr* mutants or in animals that misexpress *Hb* or *Kr,* certain GMCs are missing or take on fates associated with the factors that the mother neuroblast expressed at the time when they were born.

express a transcription factor called hunchback (hb) and the GMCs generated at this time inherit this hb. Later, the same neuroblasts turn off *hb* and express a different transcription factor, Krueppel (Kr) instead. Now, all the GMCs generated at this stage inherit *Kr* expression, but not *hb* expression. If hb is eliminated from the neuroblasts when they are making GMCs, the neuroblasts generate early GMCs that cannot make early neuron fates. Similarly, if Kr is eliminated, then later neural fates are missing. If instead, Hb is experimentally maintained in the neuroblasts at the stages when they would normally start expressing Kr, the neuroblasts keep making early GMCs (Figure 4.5) (Isshiki et al., 2001). The expression of the successive transcription factors is linked to the cell cycle, which functions as a kind of clock, since blocking the cell cycle blocks the succession and reactivating it reactivates the succession.

The expression of both spatial and temporal coordinate genes in neuroblasts is preserved in their progeny, the GMCs, and forms part of the increasingly rich inheritance of each developing neural progenitor. The ontogenetic roots of a neural progenitor can be read in the combination of transcription factors it expresses, and these factors in turn influence the cell's eventual phenotype.

ASYMMETRIC CELL DIVISIONS AND ASYMMETRIC FATE

Typically, the progeny of a cell division inherit the spatial and temporal coordinates expressed by the parental neuroblast at the time of birth. However, the parent cell often divides asymmetrically, giving intrinsic determinants to one daughter but not the other. As soon as they leave the neurectoderm behind, insect neuroblasts start dividing asymmetrically to produce two unequally sized daughter cells, a large second-order neuroblast remaining at the surface and the smaller GMC lying interiorly. How does a cell accomplish the partitioning of information selectively to one offspring and not the other? Two factors, Numb and Prospero (*Pro*), are expressed in most neuroblasts and are critical for asymmetric distribution of determinants of cell identity. At neuroblast division, these factors become localized to the smaller daughter, the GMC, where Prospero moves into the nucleus and positively influences GMC fate (Lu et al., 2000). Numb acts by inhibiting the Notch signaling pathway by binding to Notch and inactivating the transmission of a signal to the nucleus (Chapter 1). In the absence of Notch signaling, the GMC is free to move down the determination pathway.

Both Prospero and Numb are initially present throughout the entire neuroblast, so how do they get asymmetrically segregated? Figure 4.6 shows how this happens. Two proteins called Inscuteable (Insc) and Bazooka (Baz) form a complex, the Insc complex, that somehow recognizes and attaches to apical membrane of the neuroblast. The Insc complex orients the mitotic spindle along the apicobasal axis by anchoring the centrioles, which results in a vertical mitotic spindle. At the same time, the Insc complex, in conjunction with an actin-based cytoskeleton mechanism, drives the distribution of several key proteins along this vertical plane so they are inherited asymmetrically (Kaltschmidt and Brand, 2002). In particular, a cytoplasmic protein, Miranda (Mira) becomes enriched at the basal neuroblast pole such that when the cytokinesis separates the neuroblast's daughter cells, Mira is trapped in the GMC. It is Mira that binds the aforementioned determinants, Numb and Prospero, to the basal neuroblast pole and thus directs their localization to the GMC. The more apical cell does not differentiate into a GMC; rather it remains a neuroblast capable upon production of more Mira, Prospero, and Numb, to spit off another GMC at the next division. An example of the Numb protein segregating to a single daughter is shown in Figure 4.7.

An interesting example of a situation in which daughter cells adopt different fates due to asymmetric inheritance of Numb are the small sensory organs called sensilla, scattered over the body surface in *Drosophila*. The cells that compose each sensillum are usually clonal descendants of a single sensory organ precursor, SOP. The SOP cells are a bit like the neuroblasts that give rise to the CNS; they originally delaminate from the ectoderm during development in much the same way, dependent on proneural genes and Notch and Delta interactions, as described in Chapter

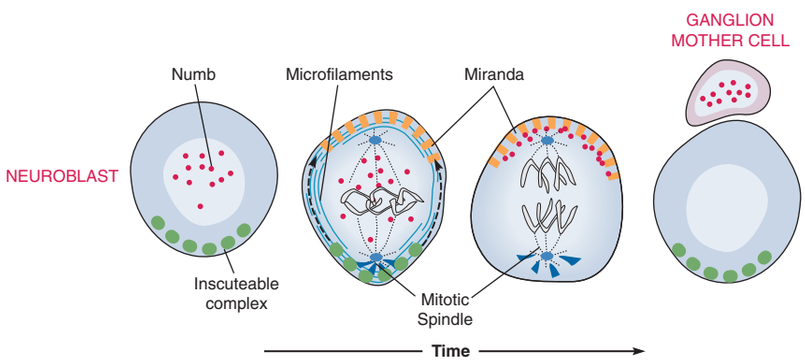

FIGURE 4.6 Control of asymmetrical cell division in *Drosophila*. The Inscuteable complex is localized to the apical pole of the neuroblast where it orients the mitotic spindle and causes the basal localization of asymmetrically localized determinants such as Miranda and Numb.

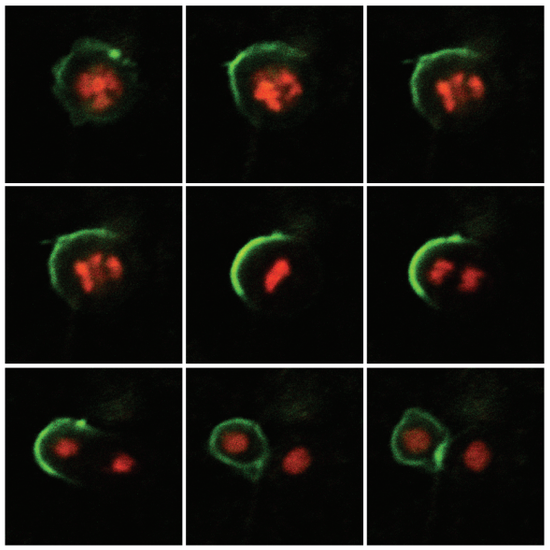

FIGURE 4.7 Frames from a time-lapse visualization of an SOP going through an asymmetrical division. The green label follows Numb, and the red label follows the chromosomes. In this sequence (courtesy of F. Schweisguth), it is easy to see Numb localized to one pole of the SOP and then inherited by a single daughter, spIIb.

1. Each specified SOP undergoes an invariant pattern of cell divisions. This division pattern has been investigated in detail for the external mechanosensilla (Guo et al., 1996; Schweisguth et al., 1996). The primary SOP (spI) for each macrochaete divides into an anterior daughter called spIIb and a posterior daughter called spIIa (Figure 4.8). SpIIa produces the outer two accessory cells: the socket cell and the shaft cell. The anterior daughter SpIIb divides into a neuron and a support cell, after first giving rise to a glial cell. These different fates arise through the reuse of the Notch signaling system during each of the cell divisions. When the SOP divides into spIIa and spIIb, these two cells interact with each other via Notch. The SpIIb fate is dominant, which is shown by the fact that if spIIb is ablated, spIIa will transform into spIIb. In Notch mutants, both cells become spIIb, and the result is no bristles or sockets, and when Notch is experimentally activated in both cells, they both turn into spIIa and there are no neurons or glia. Several intrinsic determinants, including the asymmetrically inherited Numb (see above), control the fate of spIIa versus spIIb (Figure 4.8). In this case, Numb is distributed to spIIb upon cell division. In the absence of Numb, the Notch pathway is active in spIIb, and it is transformed into spIIa; neither neurons nor support cells appear, but the sensilla form instead with double sockets and shafts. *Numb* mutants are thus insensitive to touch because the sensory bristles are uninnervated, hence the origin of the name.

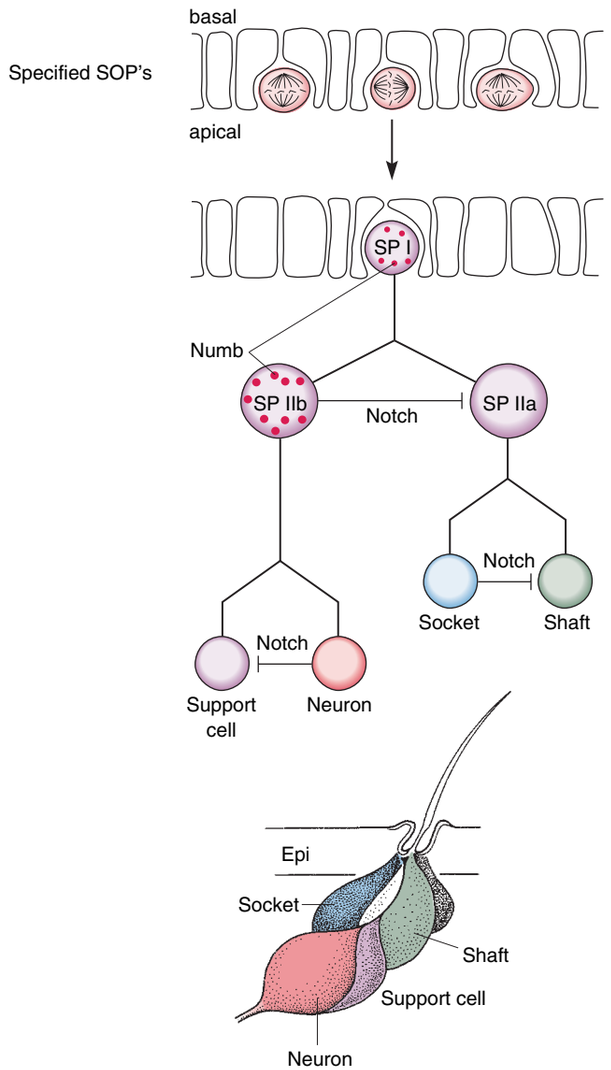

FIGURE 4.8 Lineage of a *Drosophila* external mechanosensory organ. From top to bottom: A Sensory organ precursor (SOP) enlarges and delaminates from the epithelium. It divides asymmetrically into spIIb, which inherits Numb, and spIIa, which does not. SpIIa divides again asymmetrically, as does spIIb slightly later. Notch signaling between daughters is involved in all these asymmetric divisions so that four daughter cells of the SOP have four different fates: support cell, sensory neuron, socket cell, and shaft cell.

GENERATING COMPLEXITY THROUGH CELLULAR INTERACTIONS

The compound eye of an insect is composed of a large number of identical unit eyes, called ommatidia, each with its own lens and array of cell types. This system provides a rather different example of how specific cells get their fates. Each of the 800 ommatidia

in a *Drosophila* eye possesses 8 photoreceptors and 12 accessory cells. The 8 photoreceptors (R1–R8) are specialized sensory neurons (Figure. 4.9). Among the accessory cells, there are cone cells that form the lens of each ommatidium and pigment cells that surround the photoreceptors optically shielding one ommatidium from light that enters its neighbors. What is clear in this system is that lineage is not involved in the specification of the different cell types. Experimental results have shown that there is no clear clonal relationship among the cells of the ommatidia (Ready et al., 1976). In the developing *Drosophila* retina, cell–cell interactions between the postmitotic photoreceptors and accessory cells are primarily responsible for specifying cell fate (Banerjee and Zipursky, 1990). Even after a cell has become postmitotic, it remains temporarily uncommitted to any particular differentiated fate. The mechanism controlling retinal cell fate diversification depends on the fact that the cells do not differentiate all at once, but follow a precise, reproducible temporal sequence of interactions. Thus, during late larval life, a wave of differentiation passes over the eye disc in a posterior to anterior direction (Figure 4.9). The wave front, or the position at which ommatidial differentiation begins, is morphologically visible as a morphogenetic furrow (MF), a narrow groove formed by apical constriction of the eye disc cells. As the furrow advances, cells in its wake aggregate into "rosettes" that foreshadow the regular ommatidial pattern. One cell is then singled out in each rosette. This becomes the R8 photoreceptor. The bHLH proneural gene *atonal* is turned on by the signaling protein Hh (a homolog of vertebrate *Shh*), which is expressed at the posterior tip of the eye disc (Kumar and Moses, 2000). Initially, *atonal* comes on in a continuous band of cells within the morphogenetic furrow. And this initiates a lateral inhibition mechanism that involves Notch signaling so that *atonal* expression becomes restricted to a mosaic of regularly spaced cells, which subsequently differentiate as R8. The set of well-spaced R8 cells continues to emit Hh, which signals across the MF toward the more anterior cells of the eye disc to induce the next set of R8 cells. This Hh-mediated feedback mechanism drives the morphogenetic furrow across the eye disc.

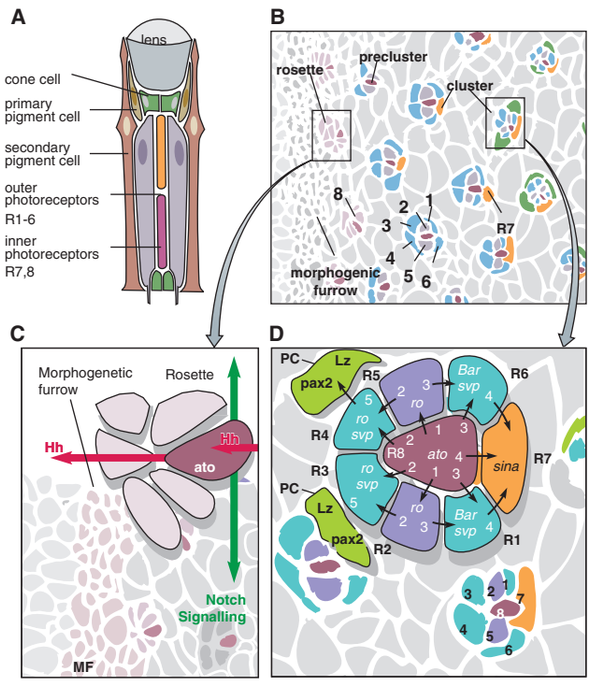

FIGURE 4.9 A. A schematic longitudinal section through an ommatidium depicting the different cell types. B. Diagram showing a surface view of part of the eye disc at a stage when photoreceptor clusters become assembled. C. A precluster in which the R8 precursor expresses *ato* in response to a previously generated hedgehog (Hh) signal. The R8 cell produces its own Hh and by this relay starts the next R8. R8s spread themselves out through the Notch lateral inhibition pathway. D. Cascade of photoreceptor determination in a developing ommatidial cluster. R8, the first cell to be determined expresses *atonal* (*ato*) and signals neighboring cells to become R2 and R5, which then express *rough* (*ro*). R2 and R5 in combination with R8 then signal the next set of neighboring cells to join the cluster of "fire" and become R3 and R4, which express *seven-up* (*svp*). On the other side, a cluster of seven cells is formed when R1 and R6 are induced to express *Bar*, *svp*, and *lozenge* (*lz*) by R2, R5, and R8. Finally R8, in combination with R1 and R6, induce the final photoreceptor R7 expressing *sevenless in absentia* (*sina*) and *prospero* (*pros*) to join. After the photoreceptors have joined, pigment cells expressing *lz* and *pax-2* are induced to join the cluster.

The first cells that join each R8 cell shortly after its determination become R2 and R5. It is believed that a signal emanating from R8 instructs the next cells to join the cluster, R2 and R5. These cells acting in combination with R8 help give the next cells that join the cluster R3 and R4 and then R1 and R6 their fates (Ready, 1989). The last photoreceptor to join the cluster is R7. Thus, ommatidial clusters incorporate cells in a manner analogous to how growing crystals incorporate molecules. That is, as new cells are added, they become neighbors of cells that are already incorporated and have particular fates. In this way, determination of a specific fate moves as a wave of crystallization across the eye disc, and so the developing *Drosophila* eye has been called a neurocrystalline array.

Each type of ommatidial cell expresses a unique set of intrinsic determinants. For example, *rough* (*ro*) is expressed in R2, R5, R3; R4, *Bar* appears in R1 and R6; *Seven-up* (*svp*) in R1, R6, R3, R4; *Prospero* (*pros*) in R7 and cone cells, *lozenge* (*lz*) in R1, R6, R7, and cone cells, and *Pax2* in cone cells only (Figure 4.9). Each of these

factors is linked to the normal differentiation of the respective cells in which it is expressed, as revealed by the fact that a particular cell type fails to develop in an eye disc that lacks the corresponding gene.

Shortly following its own determination, R8 puts out signals that activate two different signaling pathways, the Notch pathway and the Ras pathway (Freeman, 1997; Brennan and Moses, 2000). The Notch pathway, as we know, is activated by Delta. The Ras pathway is a highly conserved biochemical cascade of cytoplasmic kinases (Ras, Raf, MPK), which in this case are activated by the epidermal growth factor receptor (EGFR). R8 emits an EGF-like molecule, Spitz (Spi) that activates this receptor. Activation of these signaling cascades spreads concentrically from R8 to R2, R3, R4, and R5 and then the remainder of the ommatidial cells. The precise, temporally regulated activation of the EGFR and Notch signaling pathways assigns distinct phenotypes to the cells that join the ommatidial clusters.

The determination of the R7 cell deserves special mention in view of the pivotal role it has played in opening up the molecular-genetic study of signaling pathways. One of the first mutant screens in the field of cell determination took advantage of the fact that only R7 is sensitive to UV light. Thus, mutagenizing flies and screening for offspring that are blind to UV light yielded a fly line that lacked the R7 cell in every ommatidium and was therefore aptly called *sevenless* (*sev*) (Harris et al., 1976), (Figure 4.10). Lack of the receptor causes the cell that would normally become R7 to develop as a cone cell instead. In several followup screens, many signaling molecules in the Ras pathway activated by the Sev receptor were identified (Rubin, 1991; Hafen et al., 1994) (Figure 4.11). Among them were the *Drosophila* homologs of Ras, Raf, MEK, MAPK, and Gap1, parts of the ras signaling pathway, as well as new genes in the signaling pathway such as *Son of sevenless* (*Sos*) and *Daughter of sevenless* (*Dos*). This pathway regulates the activity of transcription factors yan and pointed (pnt) that also were identified in such screens, and these factors control the expression of genes involved in the differentiation of R7. One of the most satisfying discoveries from the screens for the sevenless phenotype was the signal called Bride of sevenless (Boss), which binds to Sev. Boss is expressed specifically in R8 cells, so when any cell expressing Sev touches R8, the Ras signaling pathway fires in this cell and it becomes R7 (Reinke and Zipursky, 1988). These experiments provide an impressive demonstration of the power of genetic screens.

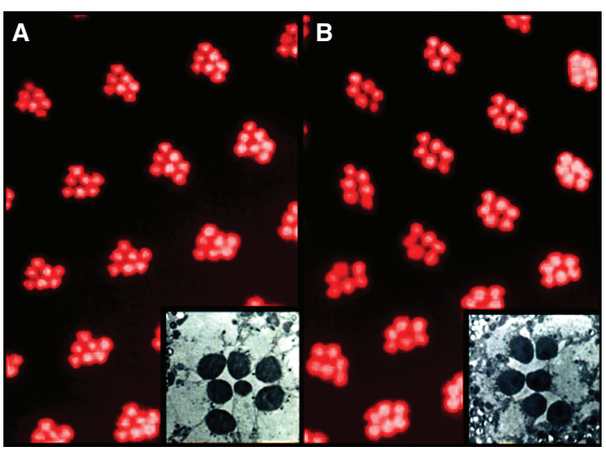

FIGURE 4.10 Photoreceptors in the eye of normal flies (A) and sevenless mutants (B). The red images show light that is piped up through the clusters of receptors in each facet. The inserts are electron micrographs through a single facet and show cross sections of the photoreceptor array. Notice that the seventh central photoreceptor is missing in each facet of the mutant eye.

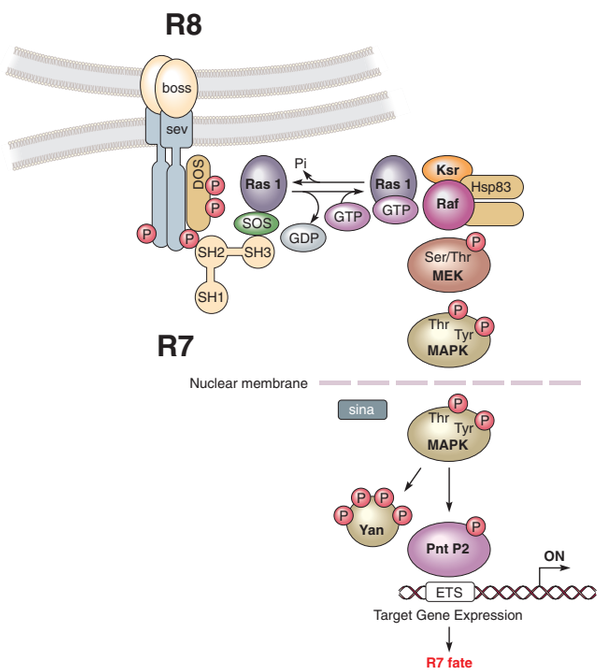

FIGURE 4.11 Components of the sevenless transduction pathway, include the seven transmembrane signaling molecule bride of sevenless (boss) which is expressed by R8, the sevenless receptor molecule (sev), and a number of downstream components of the signaling cascade such as daughter of sevenless and (Dos) and son of sevenless (Sos) and various members of the Ras Raf pathway ending in the activation of the yan and pointed genes. (Courtesy of E. Hafen)

SPECIFICATION AND DIFFERENTIATION THROUGH CELLULAR INTERACTIONS AND INTERACTIONS WITH THE LOCAL ENVIRONMENT

The vertebrate neural crest is a transient stem cell population that arises along the lateral edges of the neural plate induced by the convergence of secreted signals (notably Wnts, BMPs, and FGFs), at the juxtaposition of neural plate, lateral epidermis, and subjacent paraxial mesoderm (see Chapters 1 and 2 for details of crest induction). As described in Chapter 3, these cells migrate from their site of origin at the dorsal-most part of the neural tube, along stereotypic pathways through the rest of the embryo. In this section, we discuss how the neural crest cells become specified toward different fates as they migrate through different environments. The neural crest progenitors continue to divide as they migrate until they coalesce at their destinations. Crest cells generate a variety of cell types. Not only does the crest produce the entire peripheral nervous system, including the autonomic and sensory ganglia, and the peripheral glia (Schwann cells), but it also produces endocrine chromaffin cells of the adrenal medulla, smooth muscle cells of the aorta, melanocytes, cranial cartilage and teeth, and a variety of other nonneural components. Because of its variety of descendants, crest has been a popular model for testing the mechanisms that generate cell diversity (Le Douarin, 1982).

To test whether premigratory crest cells are committed to a particular fate, Le Douarin and colleagues transplanted the crest between different anterior-posterior positions (Figure 4.12). These experiments took advantage of the chick–quail chimeric system described in Chapter 2. The results show that crest cells acquire instructions to differentiate during their migration, as well as when they arrive at their final destination. For example, crest cells from the trunk normally give rise to adrenergic cells of the sympathetic nervous system, whereas the more anterior crest cells from the vagal region give rise to cholinergic parasympathetic neurons that innervate the gut. When vagal crest cells from quail embryos were transplanted into the trunk region of chicken embryos, the transplanted vagal crest migrated along the trunk pathways and differentiated into adrenergic neurons in sympathetic ganglia. Similarly, trunk crest cells that were transplanted to the vagal region gave rise to cholinergic neurons of the gut. Similar experiments have been done to test the competence of crest cells to form a variety of different cell types, and the general conclusion is that crest cells display great flexibility in responding to local environmental cues. It could be that each region of the crest contains a complement of specified progenitors, only some of which survive in each location, but it seems more likely, given the evidence below, that commitment to a particular fate is a multistep process of determination.

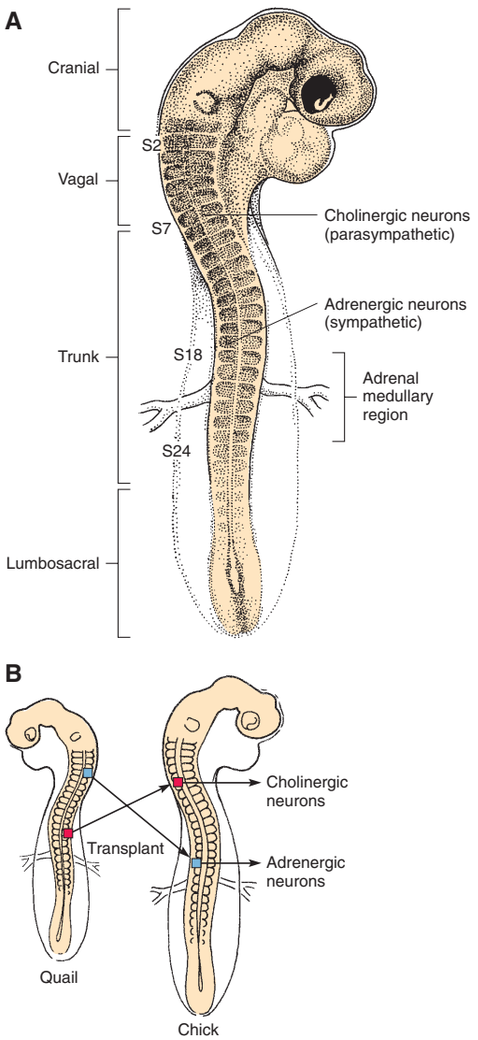

FIGURE 4.12 The environment influences the fate of neural crest cells. A. Crest cells from the trunk normally give rise to adrenergic cells of the sympathetic nervous system, whereas the more anterior crest cells give rise to cholinergic parasympathetic neurons that innervate the gut. B. When anterior crest cells from quail embryos are transplanted into the trunk region of chicken embryos, they differentiate into adrenergic neurons. Similarly, trunk crest cells that are transplanted anteriorly give rise to cholinergic neurons. (After Le Douarin et al., 1975)

Migrating crest cells become exposed to a sequence of instructive environments, each with a unique set of

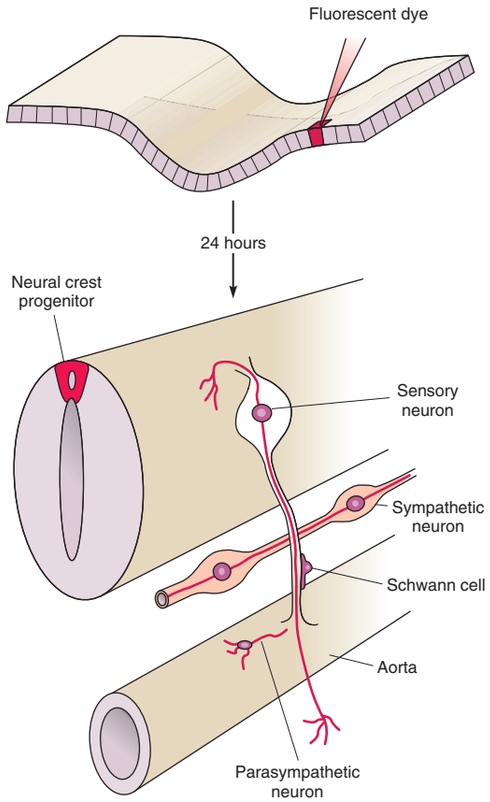

FIGURE 4.13 Fates and migration of neural crest cells. A single progenitor cell is injected with a lineage tracer, and its progeny are followed as they migrate out of the neural tube. Some may become sensory neurons, while others become Schwann cells or neurons of the autonomic nervous system. Environments these cells pass through on their migration routes influence their fate choice. (After Bronner-Fraser and Fraser, 1991)

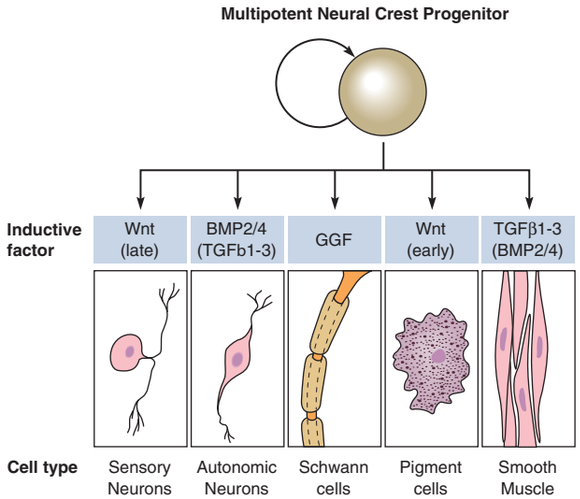

FIGURE 4.14 Different neural crest fates are promoted by a distinct set of extracellular signaling molecules. (After Dorsky et al., 2000)

factors, and the migrating cells respond to these factors and each other in a way that limits their potential. Initially, neural crest cells are multipotent, and labeling of single progenitors at the earliest stages of migration shows that these cells can give rise to a wide variety of derivatives (Bronner-Fraser and Fraser, 1988) (Figure 4.13). But as the cells migrate along particular routes, they segregate into several classes of more specialized progenitors. Thus, as development proceeds, they become more restricted. In the trunk region, an early decision separates postmigratory crest cells that will become the sensory progenitors, which remain in the somitic mesodermal region and express the proneural *bHLH* transcription factor Neurogenin (Nrgn2), from the autonomic progenitors, which do not (Lo et al., 2002). Transplantation studies with these two types of progenitors shows they can no longer make the full array of cell types. Nrgn2 is already expressed in premigratory crest cells and appears to bias but not determine them toward a sensory fate. It appears that the sensory cells of the dorsal root ganglia (DRG) inhibit other crest cells from assuming this fate. If the cells that normally make the DRGs are ablated, then later migrating crest cells will differentiate into sensory neurons (Zirlinger et al., 2002).

Experiments with purified populations of neural crest cells in culture suggest that different factors are involved in these restrictions (Figure 4.14) (Groves and Anderson, 1996; Anderson et al., 1997; Dorsky et al., 2000). It turns out that where cells encounter such factors on their migration route is very important in shaping appropriate destinies. BMPs are expressed in the dorsal aorta, where sympathetic ganglia form. BMPs turns on a program of neurogenesis in sympathoadrenal (SA) cells (i.e. cells that are either part of the sympathetic nervous system or cells that are part of the adrenal gland) by inducing the expression of the paired domain transcription factor *Phox2b* which is required for the development of all of the autonomic nervous system and the proneural *bHLH* gene *MASH1*, which is required for the expression of neuronal markers in autonomic neurons (Pattyn et al., 1999; Schneider et al., 1999). However, further cues are needed if these cells are to become mature neurons. SA progenitors plated on a laminin-containing substrate in the absence of any growth factor form short, neuron-like processes. These processes become more extensive when the growth factor FGF is added to the medium, whereas the neurotrophic factor NGF, which is needed for the survival of sympathetic neurons (see Chapter 7) has no effect at this stage. The SA progenitors are

initially unresponsive to nerve growth factor (NGF) because they do not express the NGF receptor. One of the effects of FGF is to induce the NGF receptor gene, thereby making the SA cells responsive to NGF, which stimulates their differentiation and survival as neurons (Anderson, 1993).

Sympatho-adrenal (SA) progenitors can also be isolated from the adrenal gland primordium of embryonic mammals and raised in culture; they give rise to two very different types of cells, the adrenergic sympathetic neurons and the endocrine chromaffin cells. Prior to differentiation, all SA progenitors express markers for both cell types. When SA progenitors are exposed to glucocorticoid hormone in vitro, which normally is produced in the adrenal gland, they develop as chromaffin cells. Glucocorticoids are steroid hormones that act on cytoplasmic receptors. After binding to the hormone (ligand) the receptor–ligand complex is transported to the nucleus where it acts as a transcription factor, binding to DNA and activating or repressing certain genes. In the case of the SA progenitor, glucocorticoids suppress the transcription of neuron-specific genes and activate the transcription of chromaffin cell-specific genes.

All sympathetic neurons start life producing the neurotransmitter noradrenalin. They receive the signal to be adrenergic. The Phox2b and MASH1 transcription factors induced by BMPs secreted by the aorta (Reissmann et al., 1996; Pattyn et al., 1999) (Figure 4.15) appear to be responsible for controlling the expression of tyrosine hydroxylase, a key member of the synthetic pathway for this transmitter. Many of these neurons send out axons to smooth muscle targets; these sympathetic neurons remain adrenergic throughout life. However, a few sympathetic neurons, for example, those that innervate sweat glands, switch their neurotransmitter phenotype late in development and become cholinergic; that is, they secrete the neurotransmitter acetylcholine (ACh). Neurotransmitter choice in these cells is a late aspect of cell fate that is regulated by the target (Francis and Landis, 1999). The fibers innervating sweat glands begin to turn off tyrosine hydroxylase and other adrenergic enzymes and begin to make choline acetyltransferase, the synthetic enzyme for ACh production (Figure 4.16). Evidence for the role of the sweat glands themselves in inducing the switch in phenotypes comes from transplantation experiments. Transplanting foot pad tissue, rich in sweat glands, to areas of the body that usually receive adrenergic sympathetic innervation leads to the induction of cholinergic function in the sympathetic axons that innervate the transplanted glands. Factors such as interleukin-6 are capable of causing an adrenergic-to-cholinergic switch in phenotype and have been purified from

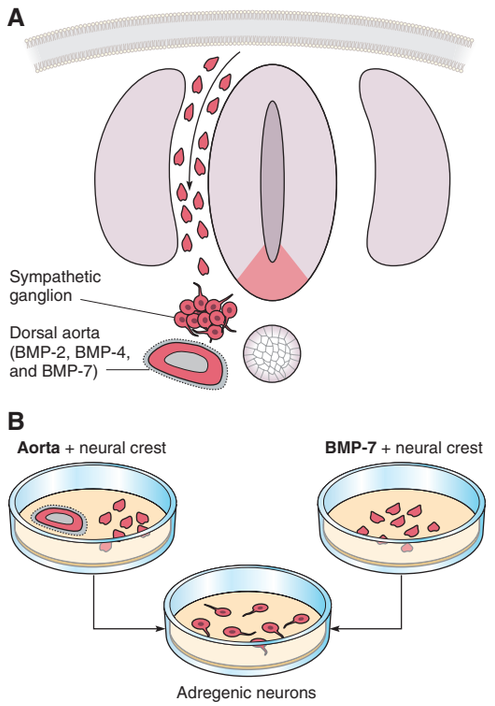

FIGURE 4.15 Control of transmitter phenotype by the aorta. A. Neural crest cells that migrate close to the aorta often become sympathetic ganglia with adrenergic neurons. The dorsal aorta is a source of BMPs. B. When neural crest cells are cultured with aorta or BMP-7, they turn on Phox2b, which activates the transcription of tyrosine hydroxylase and the cells become adrenergic neurons.

culture media, but the actual factor that operates in sweat glands to produce this effect in vivo has not yet been definitively identified. Nevertheless, these experiments make it clear that targets can retrogradely determine that transmitter type of the innervating neurons.

In the vertebrate peripheral nervous system, all glia, whether they are Schwann cells or glial support cells in the sensory and autonomic ganglia, arise from the neural crest and express *Sox10*. But the decision to express *Sox10* and commit to a glial fate happens late in the crest decision hierarchy, after the decision to be sensory or autonomic. A secreted protein called Neuregulin-1 (Nrg-1) induces crest cells to adopt glial fates (Britsch et al., 2001; Leimeroth et al., 2002) (Figure 4.17). When migrating crest cells are cultured in the absence of added Nrg-1, the majority of clones contain both neurons and glial cells, but if Nrg-1 is applied, most clones develop as pure glia. Neural crest cells express Nrg-1 only after they have migrated peripherally and coalesced into distinct masses as in the dorsal root or sympathetic ganglia. In fact, Nrg-1 is expressed only in those cells that have already started to exhibit a neuronal phenotype. The Nrg-1 receptor is expressed

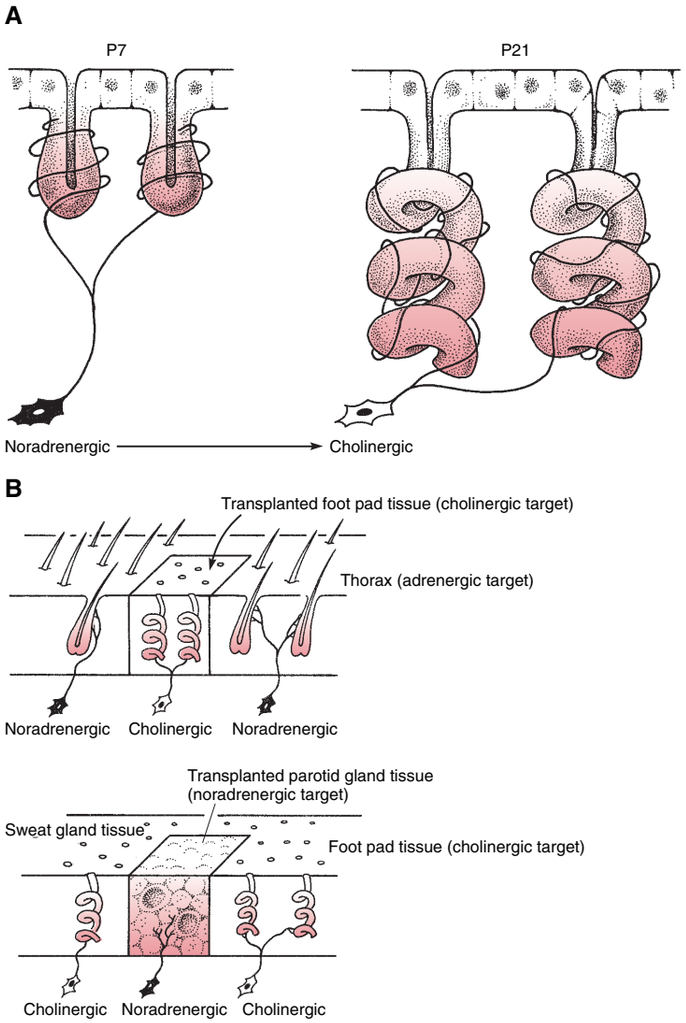

FIGURE 4.16 Control of transmitter phenotype by sweat glands in the footpad. A. A noradrenergic neuron begins to innervate developing sweat glands. As it does so, it switches and becomes cholinergic. B. Neurons that innervate hair follicles are noradrenergic, yet when a piece of footpad containing sweat glands is transplanted into hairy skin, the local neurons that innervate the transplanted sweat glands become cholinergic. Conversely, when a piece of parotid gland tissue, which is normally innervated by adrenergic neurons, is transplanted to the footpad, the local neurons that innervate it are noradrenergic. (After Landis, 1992)

by all migrating neural crest cells, so cells are sensitive to Nrg-1 as soon as they arrive at their destination but only the late cells to migrate in, those that do so after many neurons are already differentiating, see substantial amounts of Nrg-1. This is one reason why glial cells differentiate later than neurons. Another, as in the retina, has to do which Notch. Notch activation drives glial determination in crest-derived cells. As neurons begin to differentiate, they express Delta, which through Notch activation turns off proneural gene activity in neighboring cells, and thus forces them down a non-neural pathway (Morrison, 2001).

COMPETENCE AND HISTOGENESIS

The cells of the cerebral cortex are generated near the ventricular surface of the closed neural tube. As described in Chapter 3, the cell divisions of cortical neuroblasts give rise to postmitotic neurons that

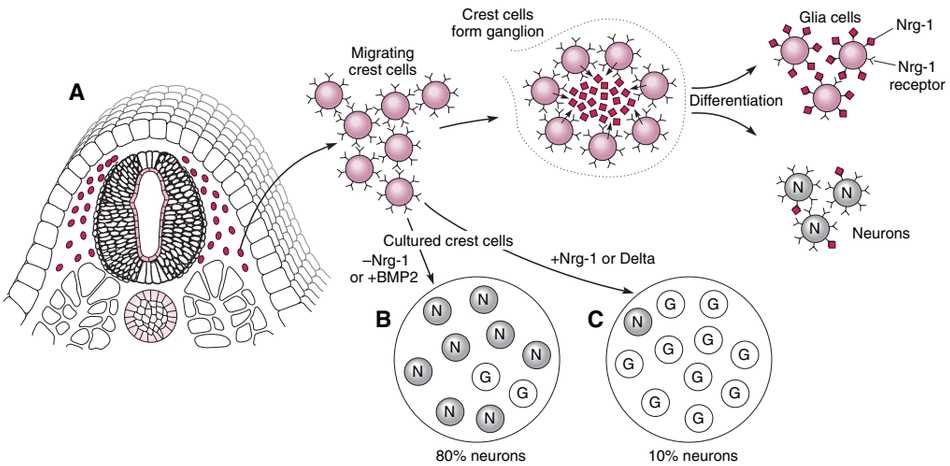

FIGURE 4.17 Specification of peripheral glia. A. Normally crest cells of the DRG produce neurons and gia (see text) and if placed in culture, crest cells give rise to both neurons (N) and glia (G). B. Removal of Nrg-1 or addition of BMP2 increase the number of neurons. C. Addition of the secreted signal Neuregulin to the medium or expression of Delta enhances the proportions of glial cells developing from the culture.

migrate along radial glia to settle in one of six different layers (Figure 4.18). In Chapter 3 we discussed the mechanisms that control the migration of these cells to their different layers; but what makes the cells of each layer distinct? The cells in the different layers of the cortex have layer-specific projection patterns. Thus, in the visual cortex, Layers 2 and 3 neurons are pyramidal cells that project to other cortical areas; Layer 4 cells are small stellate local interneurons that receive input from the thalamus; and Layers 5 and 6 cells are the largest pyramidal neurons and project to other parts of the brain, and even the spinal cord. How do the cells of a particular layer acquire their specific identities, such that they project to the appropriate targets?

Studies by McConnell and her colleagues have asked whether the progenitors of the cells destined for the deeper layers are somehow intrinsically different from progenitors of the cells destined for the upper layers, using cell transplantation in ferrets. In the ferret, Layer 6 cells are born in utero at embryonic day 29 (E29). Weeks later, cells born at P1 just after birth, are fated for Layers 2 and 3, and must migrate through Layers 6, 5, and 4 which have already formed. To test the idea that cells acquire laminar fate soon after they are born and before they migrate, cells generated in the ventricular zone of E29 ferrets were transplanted into older P1 hosts (Figure 4.19). Although their time of birth would have fated them for a deep layer, the experiments showed that many of the transplanted cells switched their fates and ended up in Layers 2 and 3, suggesting that these young cortical neurons are flexible with regard to fate (McConnell, 1988). Further studies showed that cell interactions are involved in this commitment since if the E29 cells are removed and cultured with other E29 cells for a number of hours, their deep layer fate is fixed even when challenged by transplantation to an older environment (McConnell, 1995). What about the reciprocal experiment, the transplantation of P1 precursors into E29 hosts? When P1 precursor cells were transplanted into younger brains, these cells all differentiated into Layer 2 and 3 cells, even though the host neurons around them were differentiating as Layer 6 neurons. Thus, these P1 cells seemed to have lost the competence to differentiate as deep layer cells (Figure 4.19).

This loss of competence appears to be a sequential process, as illustrated by the transplantation of progenitors in the middle stages of cortical development. When the progenitors of Layer 4 neurons born at E36 are transplanted into older P1 brains, in which Layers 2 and 3 were being generated, they also generate Layer 2 and 3 neurons. However, when E36 progenitors are transplanted into a younger E29 hosts, they show a restricted potential ending up in Layers 4 and sometimes 5, but not Layer 6 (Desai and McConnell, 2000) (Figure 4.19). These results suggest that environmental cues can influence precursors to produce neurons of different cell types, but that the competence of these precursors becomes increasingly restricted over time. Thus, they can respond to an older environment but not a younger one.

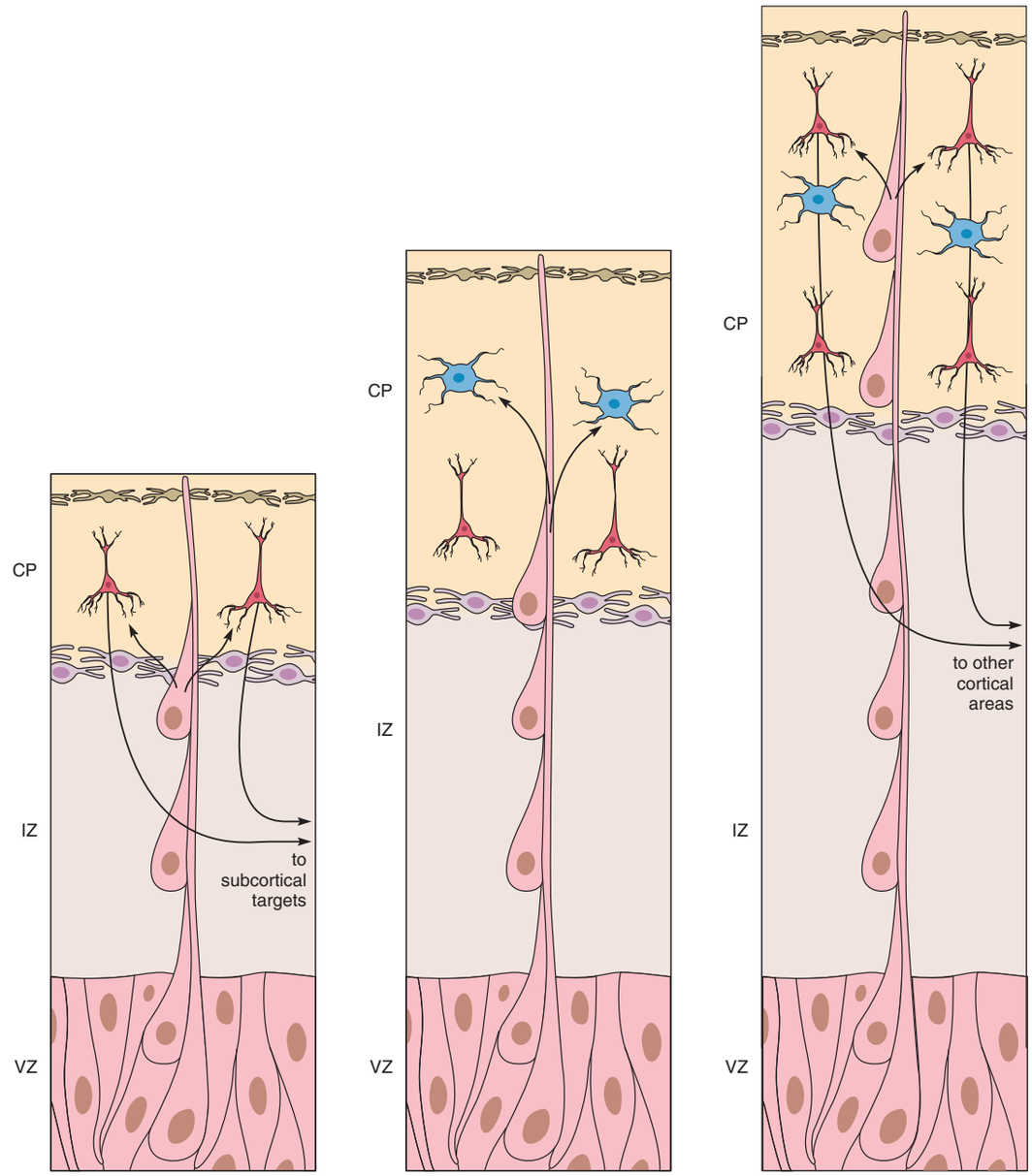

FIGURE 4.18 This figure picks up where Figure 3.15 leaves off, after the birth and migration of the Cajal-Retzius cells and the Subplate cells. A. The next neurons to be generated in the cortex are the pyramidal neurons of the deep layers, V and VI, whose axons project to subcortical targets. B. The next neurons to be born are the local interneurons in Layer IV of the cortex. C. Finally, the pyramidal cells of the upper layers, II and III, are generated. They send axons to other cortical areas.

THE INTERPLAY OF INTRINSIC AND EXTRINSIC INFLUENCES IN HISTOGENESIS

The vertebrate retina, like the mammalian cortex, is organized into layers, and there is a clear histogenetic pattern of cellular birth. To understand cell determination in this system, it is necessary to know the basic cell types. In addition to photoreceptors, which include rods and cones, the vertebrate retina contains interneurons, including horizontal cells, bipolar cells, amacrine cells, and projection neurons called retinal ganglion cells (RGCs). The RGCs are the output

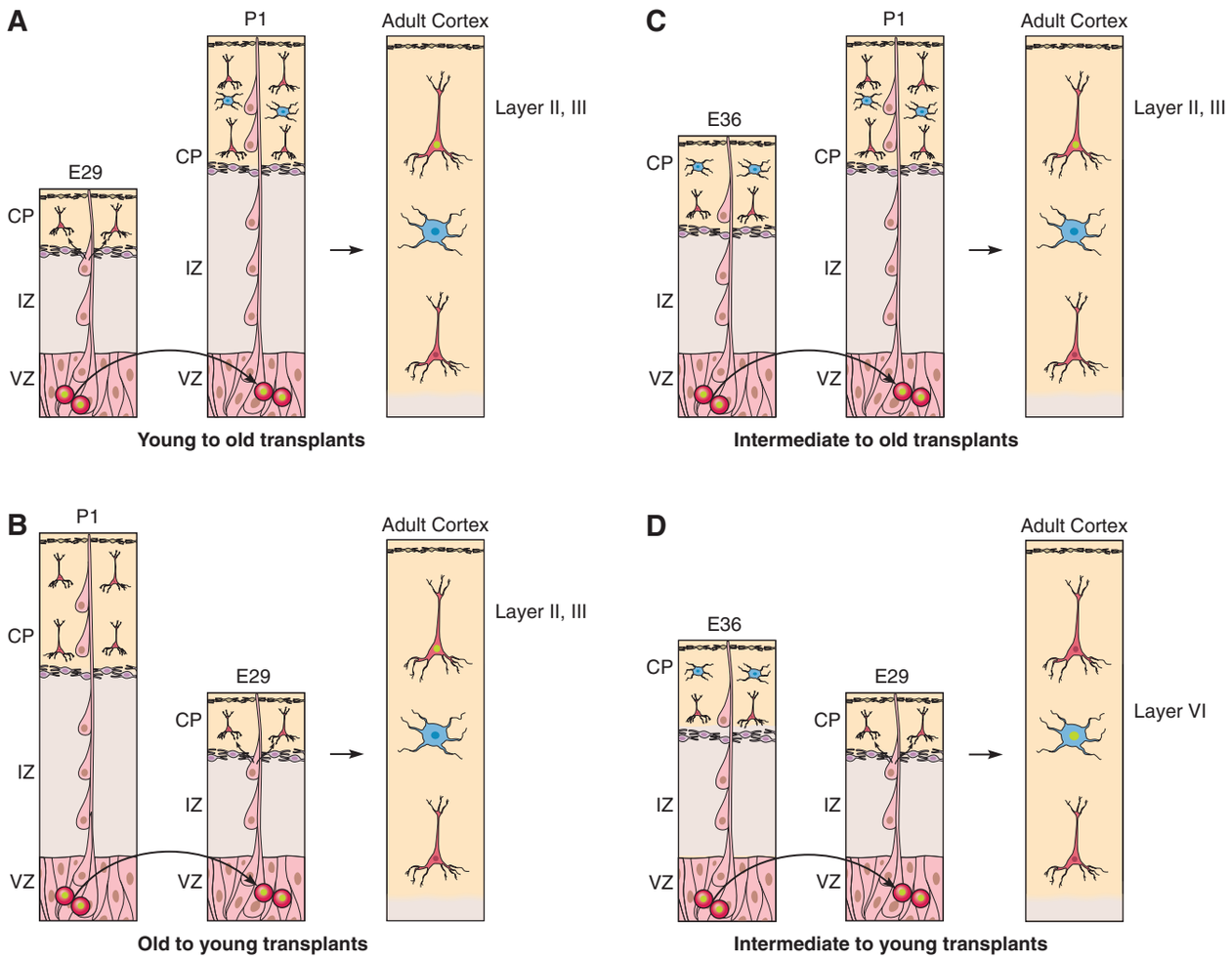

FIGURE 4.19 Progressive restrictions in fate determination in the cerebral cortex. A. Transplantation of cells from the VZ of an E29 ferret to a P1 ferret leads these cells to change from a deep layer (early) to a superficial layer (late) fate. B. But when P1 cells are transplanted to E29 hosts, they retain their superficial layer fates. C. Intermediate E36 generated cells normally destined for Layer IV can assume later fates when transplanted into an older host. D. But they cannot assume younger fates when transplanted into younger hosts.

neurons of the retina, and their axons form the optic nerve that relays the visual image to the brain. Thus, there are just six basic types of neuron in the retina and one type of intrinsic glial cell, called the Müller cell. Vertebrate retinal cells develop from a population of neuroepithelial progenitors, which produce this diversity of neurons and glia. Injection and infection of single retinoblasts with heritable markers produces clones of mixed, and seemingly random, cellular composition (Turner and Cepko, 1987; Holt et al., 1988; Wetts and Fraser, 1988), indicating that, as in the *Drosophila* retina, lineage is not the dominant mechanism of fate determination in these cells (Figure 4.20).

As in the cortex, it seems that the competence of retinoblasts becomes more restricted as development proceeds (Adler and Hatlee, 1989; Reh and Kljavin, 1989; Livesey and Cepko, 2001). During normal development, RGCs are born and differentiate first, next are amacrines, horizontal cells, and cones, and the later born neurons are rods and bipolar cells (Sidman, 1961; Cepko et al., 1996). The final cell type to be born is the Müller glial cell (Figure 4.21). This pattern is largely, though not entirely, preserved among vertebrates. To test the idea of changing competence, progenitors at various stages of retinal development are forced to differentiate in culture. If progenitor cells are isolated at a time when RGCs are normally born, they tend to turn

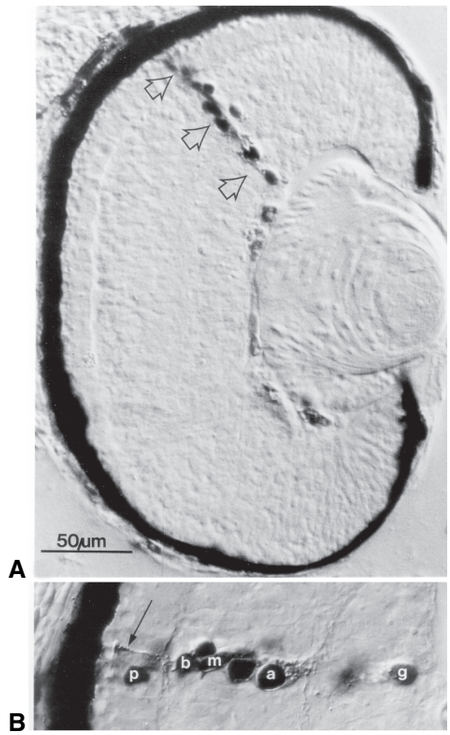

FIGURE 4.20 Clone of cells in the *Xenopus* retina. A. Daughters of a single retinal progenitor injected with horseradish peroxidase form a column that spans the retinal layers and contributes many distinct cell types. B. p, photoreceptor; b, bipolar cell; m, Müller cell; a, amacrine cell, g, ganglion cell.

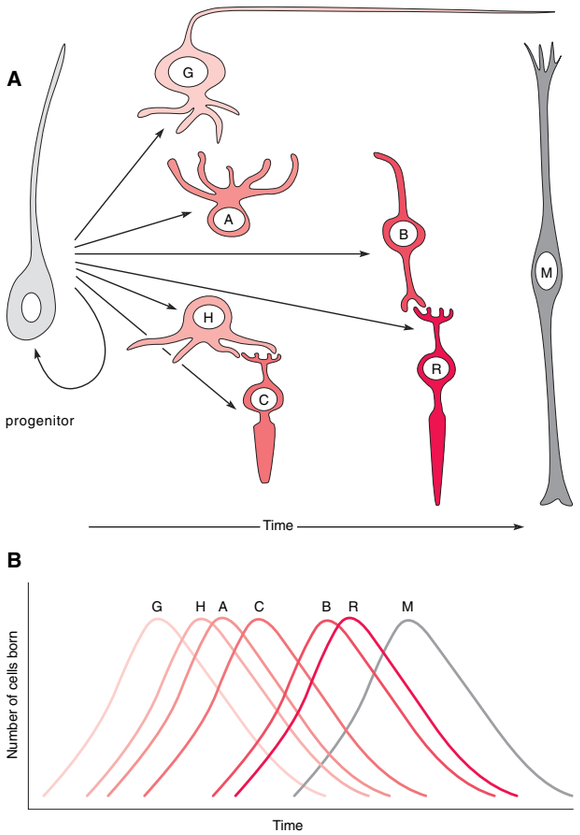

FIGURE 4.21 Determination in a vertebrate retina. A. A progenitor cell in the neuroepithelium divides several times and gives rise to clones of cells that contain all the major cell types of the retina, including Ganglion cells G, Amacrine cells A, Horizontal cells H, Bipolar cells B, Rods R, Cones C, and Müller cells M. These cells tend to be born at different developmental times, indicating a rough histogenetic order that is shown in (B).

into RGCs in culture. Progenitors isolated at a later stage tend to become rods (Watanabe and Raff, 1990) (Figure 4.22). And while it is not impossible, it is certainly more difficult for older progenitors to assume early fates than it is for early progenitors to assume late fates. This is shown by heterochronic experiments. If retinal cells that are born at the stage when RGCs are normally being born are labeled and mixed together into an aggregate and cultured in vitro, the labeled cells still differentiate into RGCs and few become rods. If, however, they are mixed with an excess of retinal cells that are several days older, the same cells have a higher probability of becoming rods (Watanabe and Raff, 1990). The other direction is much harder; that is, few late progenitors choose RGC fates, even when mixed with an excess of cells from earlier retinas (James et al., 2003).

The bHLH and homeobox transcription factors look as though they are intrinsic factors that help specify retinal cell fate. Among the homeobox factors, Chx10 is involved in bipolar fate, Prox1 in horizontal cell fate, and BarH1 in RGC fate. Among the *bHLH* genes, *Ath5* is absolutely critical RGC fate. The example of *Ath5* is particularly interesting. *Ath5* is turned on transiently in retinal precursor development. In animals where *Ath5* is never expressed, the RGCs simply do not arise. Yet other retinal cells are formed in *Ath5* knockouts. This suggests that when this factor is downregulated, retinal precursors are no longer competent to make RGCs. Moreover, when retinal progenitors are forced out of the cell cycle by the expression of cell-cycle inhibitors, at a time when they express *Xath5*, they have an increased tendency to make RGCs, suggesting that the retinal progenitors are like the neuroblasts of the *Drosophila* CNS, which go through a succession of transcription factors. The external factors that influence competence may then turn out to be factors that influence cells to exit the cell cycle and differentiate rather than factors that influence the expression of specific *homeobox* or *bHLH* genes directly.

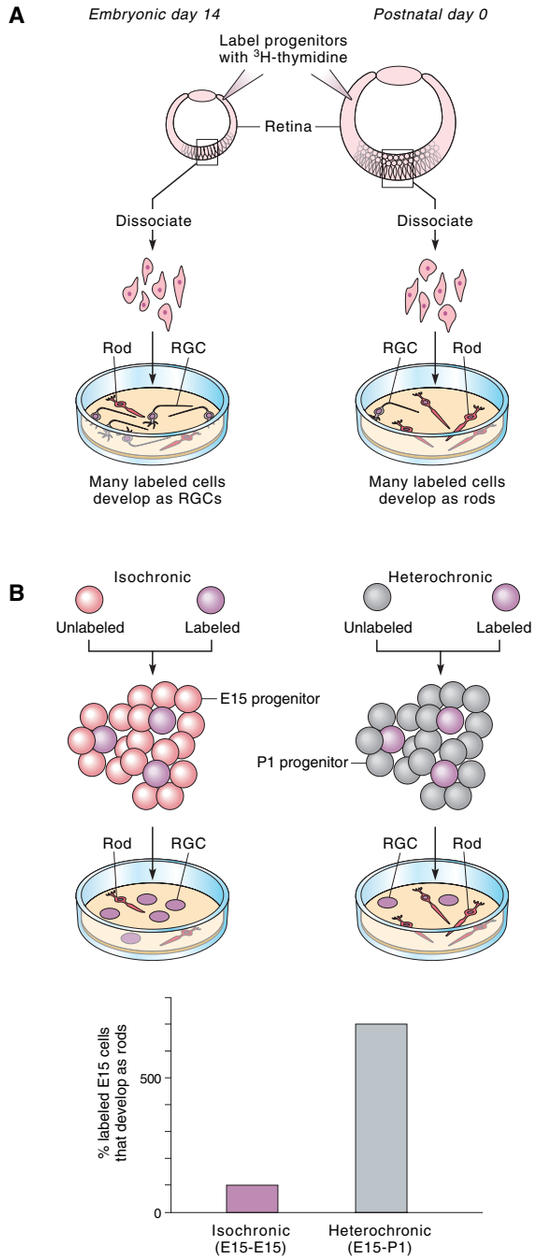

FIGURE 4.22 A. Birthdate and cell fate. Cells born on E14, even if dissociated into tissue culture, tend to differentiate into Retinal Ganglion Cells (RGCs), while cells born on P0, when dissociated, tend to differentiate into Rods. B. Early cell fate in the vertebrate retina is flexible and influenced by extrinsic factors. E15 cells labeled with thymadine and mixed with other E15 cells (isochronic) will not tend to differentiate as rods, while the same cells, if mixed with P0 cells (heterochronic) will. (After Watanabe and Raff, 1990)

Although the intrinsic transcription factors like Ath5 are critical for directing progenitors to specific fates, as noted above, cellular interactions can control the process to some extent. As we have already seen in examples from *Drosophila,* Notch signaling is an important mediator of cellular interactions that allow cells to choose different fates. Notch signaling is also very important in helping specify fate in the developing vertebrate retina. When Notch is overactive over an extended period, cell differentiation is delayed, and as a result, most retinal cells take late fates such as Müller glia (see above). Alternatively, if Notch signaling is blocked through the misexpression of dominant negative mutant genes, most retinal cells take on early fates such as RGCs. The idea proposed for Notch function in the vertebrate retina is that it allows only a certain number of cells to differentiate at any one time. This is critical when cells are making decisions based on the cues they receive in a rapidly changing environment. Thus, fates appear that are appropriate for the time at which they differentiate (Dorsky et al., 1997; Henrique et al., 1997). If all the cells were permitted to differentiate at the same instant, they might do so in more or less the same environment, and they might all choose the same fate!

Findings such as the above provide strong insights into the relationship between histogenesis and cell fate, as cells that pull out of the cell cycle at times when they express specific transcription factors may tend to produce fates based on these factors. A variety of external growth factors influence cell-cycle progression by affecting the expression of cell-cycle components (see Chapter 3). In the retina, as in many other parts of the CNS, glia are the last cells to pull out of the cell cycle. In the *Xenopus* retina, this is by virtue of an accumulation of a cell-cycle inhibitor called p27Xic1. If a retinal precursor expresses a proneural gene, like the atonal homolog Ath5, it may leave the cell cycle and differentiate as a neuron, but Notch activation in the precursor antagonizes the expression of the proneural genes like *Ath5* and allows precursor to build up p27Xic1. Interestingly, p27Xic1 is a bifunctional molecule. It has a cyclin kinase inhibitor domain to take the cells out of the cell cycle, and it has a separate Müller glial determination domain. The p27Xic1 forces the last dividing retinoblasts, those that have not been determined to become neurons, both to leave the cell cycle and to become Müller cells (Ohnuma et al., 1999). Thus, the neurogenic signaling pathway and the factors that regulate the cycle in the vertebrate retina are basic regulatory mechanisms that can be used to generate neuronal diversity by affecting the timing of differentiation in the changing external environment, linking histogenesis with determination.

INTERPRETING GRADIENTS AND THE SPATIAL ORGANIZATION OF CELL TYPES

The vertebrate spinal cord is composed of a variety of cell types, including motor neurons, local interneurons, and projection neurons. The embryonic spinal cord even contains a set of sensory neurons, called Rohon–Beard cells. These cells die by adult stages, and sensory input to the spinal cord is supplied by dorsal root ganglion neurons. The spinal cord primordium begins as a rectangular sheet of neural plate epithelium centered above the notochord. Lineage tracing experiments show that cells in the lateral edges of plate tend to give rise to Rohon–Beard cells and dorsal interneurons, while cells in the medial plate tend to give rise to motor neurons and ventral interneurons (Hartenstein, 1989) (Figure 4.23). Occasional clones are composed of different cell types, so it is thought that local position, rather than lineage, is involved in the generation of neuronal diversity in the spinal cord.

As we saw in Chapter 2, tissues outside the nervous system often provide critical signals that influence development within the CNS. In that chapter, we also reviewed the evidence that the notochord played a key role in establishing the dorsal-ventral axis of the neural tube, by providing a source of Shh. In this section, we will see how the same signal from the notochord is critical for spinal neuron differentiation acting as an organizing center for the induction of cell fate (Jessell et al., 1989). As we saw in the previous chapter, Shh secreted by the notochord induces the neural plate cells that are directly above to become the floorplate of the spinal cord. Once the floorplate has been induced in the ventral spinal cord, Shh signaling is relayed by the floorplate into the ventral neural tube. The ventrolateral region of the tube that gives rise to motoneurons sees a fairly high dose of Shh, while further dorsally, where interneurons develop, the dose of Shh is lower. In response to this single gradient of Shh, several different neuronal types are generated. The most ventral neurons require the highest doses of Shh, and successively more dorsal ones require correspondingly less. When Shh is missing as in a knockout or is antagonized with an antibody, there is no floorplate, nor any ventral neuronal type in the spinal cord. When intermediate regions of the cord are exposed to higher levels of Shh, cells there take on more ventral fates, such as motor neurons.

How do cells at different dorso-ventral levels interpret their exposure to different levels of Shh to acquire different fates? Jessell and colleagues (Jessell, 2000) have shown that particular threshold levels of Shh turn on some *homeodomain* genes (Class II) and turn off

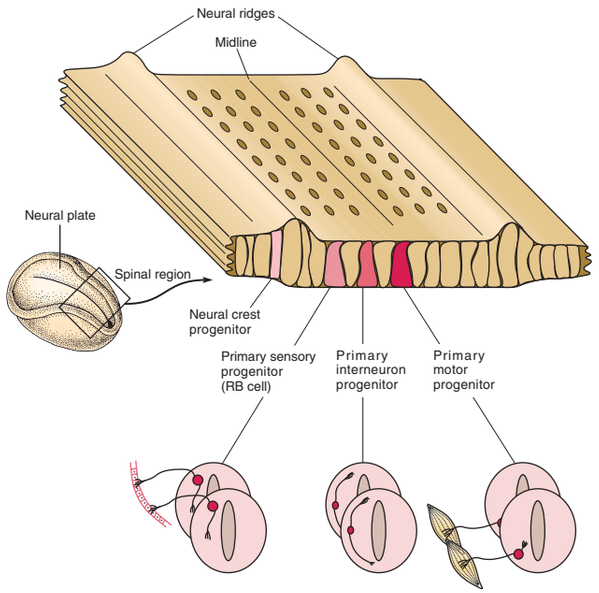

FIGURE 4.23 Rows of primary neurons in the neural plate. The most lateral row gives rise to sensory neurons (Rohon Beard Cells), the middle row gives rise to interneurons, and the most medial row gives rise to primary motor neurons. (After Hartenstein, 1989)

others (Class I). Thus, the read-out of the Shh level is first registered in neural tube neurons by expression of specific homeodomain proteins, which are either turned on or off at particular Shh thresholds (Figure 4.24). In this way, the ventral boundaries of Class I expression and the dorsal boundaries of Class II expression set up unique domains. The boundaries between these domains are sharpened through cross-repression of the two classes of genes. For example if the ventral border of the Class I *Pax-6* gene overlaps the dorsal boarder of the Class II *Nkx2.2* gene, cross-repression sets in, so that only one of these genes is expressed in any particular domain. By this process, specific domains uniquely express particular combinations of Class I and Class II homeodomain transcription factors. This results in spatial coordinates along the DV axis that are very similar to those used in setting up the mediolateral coordinate genes in *Drosophila*. Indeed, the *Drosophila* mediolateral coordinate genes share homologies with these vertebrate dorso-ventral neural tube genes, suggesting a conserved coordinate system.

Motor neurons arise from the domain that uniquely expresses Nkx6.1 but not Irx3 and Nkx2.2. Nkx6.1, unhindered by the repressive activities of these other factors, turns on OLIG2, a bHLH transcription factor that is required for motor neuron differentiation. OLIG2 in turn activates the expression of motor neuron specific transcription factors such as Mnr2, Hb9, Lim3, and Isl1/2. Once expressed, Mnr2 can regulate its own

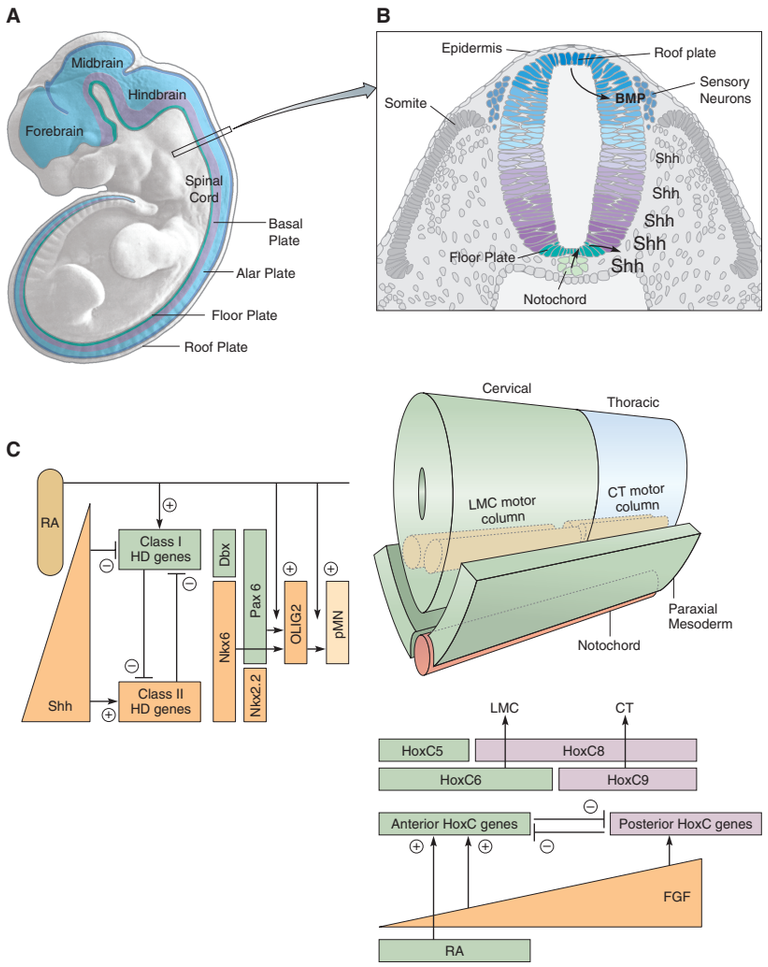

FIGURE 4.24 Specification of motor neurons in the vertebrate spinal cord. A. The neural tube, shown here for a mouse, is subdivided into four longitudinal domains: the floorplate, basal plate, alar plate, and roof plate. Motor neurons are derived from the basal plate. B. Schematic cross section of the neural tube. The notochord, which is located underneath the floorplate, releases *Sonic hedgehog (Shh)* which induces the floorplate to release its own *Shh*. This forms a gradient in the neural tube with high concentrations ventrally and low concentrations dorsally. BMP molecules released from the dorsal epidermis and roof plate form an opposing gradient. C. (Left) A gradient of *Shh* emanates from the notochord and floorplate; threshold levels of *Shh* turn on Class II HD genes. Retinoic acid (RA) expressed by the paraxial mesoderm induces the expression of Class I HD genes that are turned off more ventrally by threshold levels of *Shh*. Class I and Class II HD transcription factors cross-repress each other, creating sharp definitive boundaries at different dorso-ventral levels in the cord. Thus the boundary between Dbx and Nkx6 is more dorsal than the boundary between Pax6 and Nkx2.2. Between these boundaries, the OLIG2 *bHLH* transcription factor necessary for motor neuron specification is turned on by the concerted action of RA, Nkx6 and Pax6. OLIG2 and RA are necessary for the expression of motor neuron differentiation genes of the pMN family. (Below) A gradient of *FGF8* emanates from the mesoderm. High levels of *FGF8* turn on more caudal *HoxC* genes, whereas RA and low levels of *FGF8* turn on rostral *HoxC* genes. Rostral and caudal *HoxC* transcription factors cross-repress each other, creating sharp definitive boundaries at different rostrocaudal levels in the cord. The boundary between *Hoxc6* and *Hoxc9* establishes the boundary between the LMC of the cervical cord and the CT of the thoracic cord.

expression and is sufficient to drive spinal progenitor cells down a motor neuron pathway. This is shown by experiments in which motor neurons arise dorsally when Mnr2 is expressed ectopically in dorsal progenitors.

The motor neurons in the spinal cord are organized into functional columns that project to different muscle groups in the mature animal along the anterior to posterior axis of the body. Thus, in the cervical region is the Lateral Motor Column (LMC) that innervates forelimb muscles and in the mid-thoracic region is the Column of Terni (CT) that innervates the sympathetic chain. The anterior to posterior patterning of motor

neurons into motor columns is accomplished in response to a gradient of FGF8 (high FGF8 posteriorly to low FGF8 anteriorly) secreted by the paraxial mesoderm. This gradient establishes domains of different *Hox* genes (Dasen et al., 2003). The anterior *Hox* genes inhibit the expression of the posterior *Hox* genes, and vice versa, so that sharp borders are established. The boundaries between these domains establish the boundaries of the different motor columns. We can appreciate that this logic is strikingly similar to that governing the positioning of the motor neurons in the ventral region of the spinal cord. Thus, exposure to gradients in both axes leads to the differential expression of *homeobox* genes that through cross-repression establish sharp borders and different motor columns in the ventral spinal cord (Figure 4.24).

These motor columns can be further divided into pools that innervate specific muscles. In zebrafish, each spinal segment has just three primary motor neurons: RoP, MiP, and CaP (for rostral, middle, and caudal primary, respectively; Figure 4.25). CaP innervates ventral muscle, RoP innervates lateral muscle, and MiP innervates dorsal muscle. If these motor neurons are transplanted to different positions a few hours before they begin axonogenesis, they seem to switch fate: for example, CaP transplanted into the RoP position can innervate lateral instead of ventral muscle (Eisen, 1991). These results suggest that the position of the cell soma specifies the axonal projection of the different primary neurons.

Motor pools that innervate individual muscles are distinguished by the expression of distinct members of

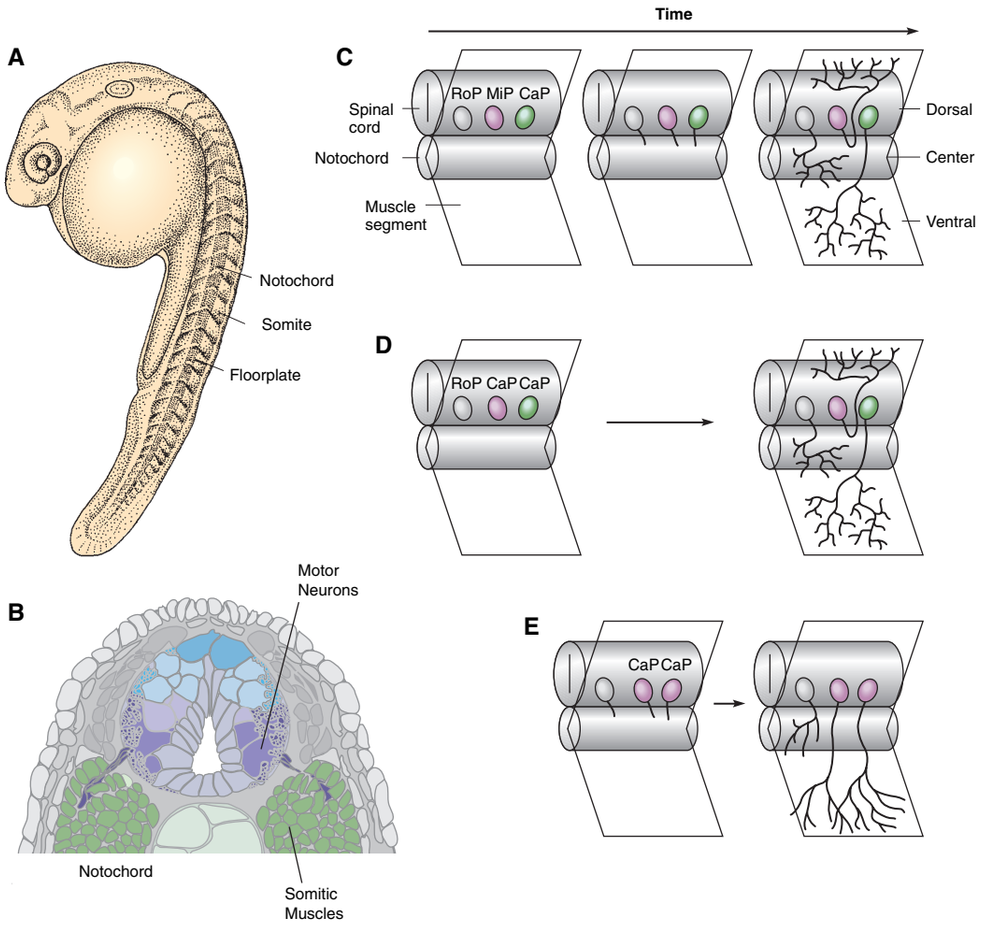

FIGURE 4.25 Position determines primary motor neuron identity in zebrafish. A. Zebrafish embryo at about 1 day old. B. Schematic cross section showing the location of the primary motor neuron and the somites that give rise to the axial musculature. C. The rostral (RoP), middle (MiP), and caudal (CaP) primary motor neurons of a single segment develop over a course of about 24 hours. D. If CaP is transplanted to the MiP position before axonogenesis begins, it develops a MiP axonal projection. E. However, if the transplant is done several hours later after the axons have begun to grow, the axonal fates are fixed.

the ETS family of transcription factors. For example, the *ETS* gene *ER81* is expressed in the motor neurons that innervate the limb adductor muscle in chicks, while the iliotrochanter motor neurons express the *PEA3 ETS* gene. Recent work suggests that these *ETS* genes regulate the expression of cell adhesion and axon guidance factors that help motor neurons recognize their targets (Price et al., 2002). Interestingly, the sensory afferents that innervate the stretch receptors in particular muscles express the same *ETS* gene as the motor neurons that innervate those muscles (Lin et al., 1998), and this helps the sensory neuron axons find the dendrites of these motor neurons completing the monosynaptic stretch reflex (Chen et al., 2003). Limb ablation studies show that signals from the periphery, perhaps from the muscles themselves, help establish the pattern of *ETS* gene expression in the sensory neurons that innervate particular muscles (Figure 4.26).

As described in Chapter 3, there are two main types of glial cells in the brain, the astrocytes and the oligodendrocytes. The oligodendrocytes are the cells of the vertebrate CNS that produce myelin sheaths around axons. While early views of CNS development proposed that these cells could arise throughout the CNS, we now know that these cells arise from relatively restricted domains in the ventral regions of the brain, and then they migrate to the axon tracts throughout the brain (Figure 4.27). Several years ago, it was discovered that a specific growth factor, PDGF, is a mitogen for the oligodendrocyte precursors in the spinal cord. Subsequent work demonstrated that the receptor for this mitogen, the PDGFα, is specifically expressed in a restricted domain of the neural tube that gives rise to the oligodendrocytes. It was therefore not too surprising that when several groups reported cloning oligodendrocyte-specific transcription factors, Olig1 and Olig2, these factors were expressed in the same ventral domain as the PDGF receptor. But what was very surprising was that the domain of Olig1/2 expression was the same domain from where motoneurons were arising! How do the same progenitors make both motoneurons and oligodendrocytes? The answer lies again in the changing competence of the progenitors. The cells that express Olig1 initially also express neurogenin2 (Nrgn2), a transcription factor that is similar to the other proneural *bHLH* transcription factors (Kessaris et al., 2001; Zhou et al., 2001). During the time when the cells in this region of the spinal cord express both Nrgn2 and Olig1, these progenitors generate motoneurons. During the same time that these cells are making motoneurons, the zone of olig1 is dorsal to the zone of Nkx2.2 expression. Later in development, the zone of Nkx2.2 moves dorsally to overlap with the Olig1/2 domain, while the Nrgn2 expression domain moves further dorsally and now no longer overlaps with the Olig1/2 expression domain. At this point in time, the progenitors that express both olig1/2 and nkx2.2 now start making oligodendrocytes. Experimentally misexpressing olig2 along with ngn2 causes the progenitors to produce motoneurons, whereas misexpressing olig2 and nkx2.2 causes the progenitors to produce oligodendrocytes. In olig2 knockout mice, neither oligodendrocytes nor motor neurons develop.

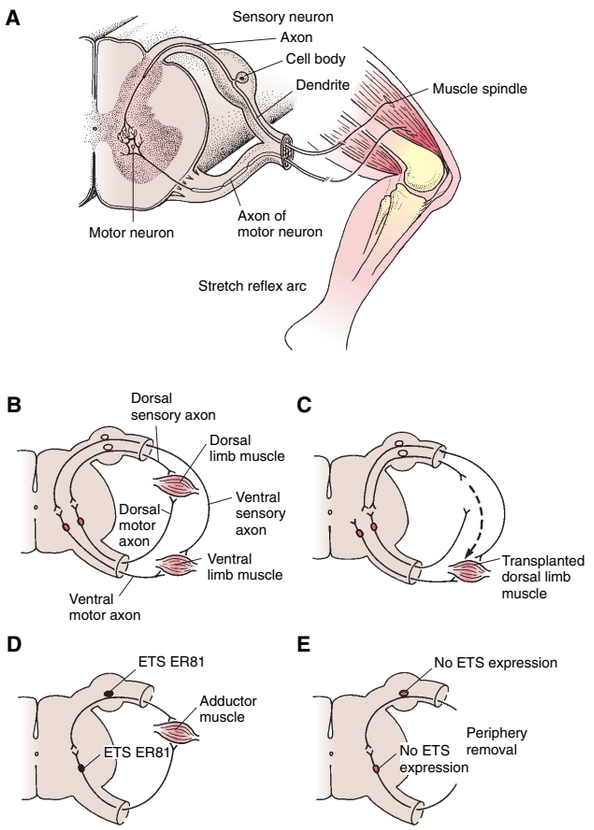

FIGURE 4.26 Matching sensory motor connectivity determined by muscles. A and B. Spindle afferents terminate on homonymous motor neurons. C. If the sensory fibers that normally innervate ventral muscles are forced to innervate dorsal muscles, they switch synaptic partners to the motor neurons that normally innervate dorsal muscles. D. Different muscles seem to induce the particular ETS molecules on both the motor and sensory neurons that innervate it. Thus, the motor and sensory neurons that innervate the Adductor muscle express the ETS ER81 molecule. E. If the peripheral muscles are removed, the motor and sensory neurons no longer express ETS molecules. (After Lin et al., 1998)

SUMMARY

In this chapter we have looked at the cellular determination in several different systems and have seen

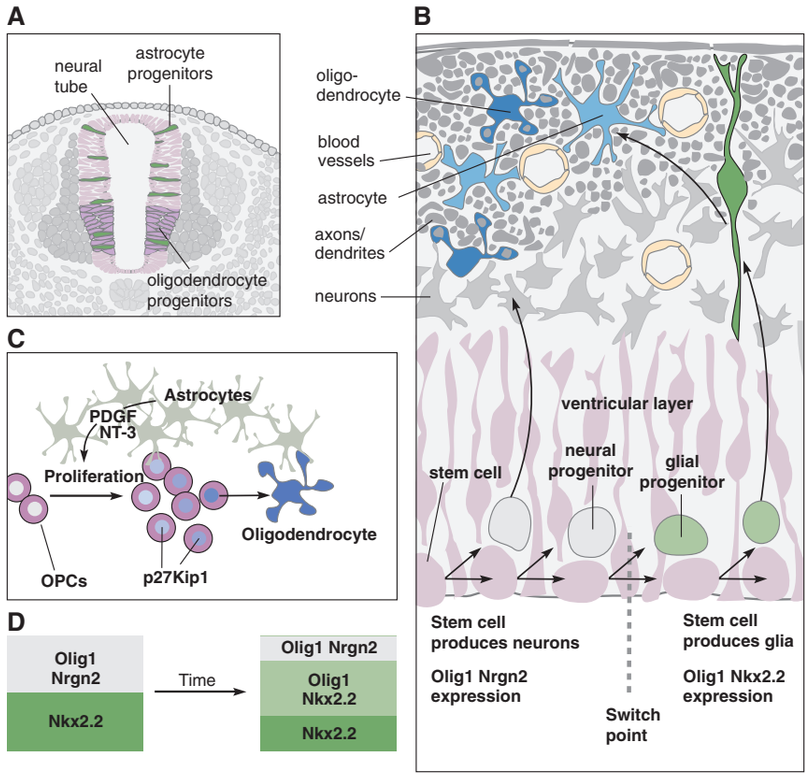

FIGURE 4.27 Glial cell development in vertebrates. A. Two main types of glial cells, oligodendrocytes and astrocytes, are formed in the neural tube. Astrocyte progenitors are distributed at all levels, whereas oligodendrocyte progenitors derive from the ventral neural tube. B. Oligodendrocytes form processes that wrap around axons and give rise to the myelin sheath. Astrocyte processes connect to capillaries and neurites. Glial progenitors and neural progenitors are derived from the same pool of stem cells that divide in the ventricular layer of the developing neural tube (bottom of B). At early stages, a stem cell generates neuroblasts. Later it undergoes a specific asymmetric division (the "switch point"), at which it changes from making neurons to making glia. C. In a culture system, optic nerve-derived oligodendrocyte progenitors (OPCs) depend on secreted signals from astrocytes. PDGF and NT-3 maintain OPC proliferation. In the absence of these factors, OPCs stop dividing and differentiate. The internal clock that determines when an OPC stops dividing depends on the level of the p27Kip1 protein, a cell cycle inhibitor that builds up over time and finally drives the cells to exit the cell cycle. D. The dorsally shifting expression of Nkx2.2 causes a change in gene expression in the progenitor cells driving the switch point (see text).

common themes such as successive restrictions in potency and potential as progenitor cells develop and divide. There is immense variation in the role of lineage versus environment in neuronal determination, with the general rule that invertebrates are more dominated by lineage mechanisms, while vertebrates are more dominated by diffusible signals and cellular interactions. Each determination pathway, however, usually brings its own mix of lineage-dependent and lineage-independent mechanisms. Of the transcription factors, bHLH factors of the proneural class help tell cells to become neurons and are antagonized by the Notch pathway which favors late differentiation of glia. Homeobox and paired domain transcription factors are often used to restrict neurons to certain broad classes linked to their position or coordinates of origin. Finally, POU, LIM, and ETS domain transcription factors may restrict cellular phenotypes even further. Of the signaling molecules, we find a particularly important role for the Notch pathway, but also important roles for BMPs, FGFs, and Hedgehog proteins. The last phases of determination involve each neuron interacting with its synaptic targets, which may provide the final differentiative signals for the maturing neuron. At the end of this process, the neuron becomes an individual cell with its own biochemical and morphological properties and its unique set of synaptic inputs and outputs.