Genesis and migration 3

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The human brain is made up of approximately 100 billion neurons and even more glial cells. The sources of all these neurons and glia are the cells of the neural tube, described in the previous chapters. Neurogenesis and gliogenesis, the generation of neurons and glia during development, is collectively also called histogenesis. Once the neurons and glia are generated by the progenitors during development, they almost always migrate over some distance from their point of origin to their final position. This chapter describes the cellular and molecular principles by which the appropriate numbers of neurons and glia are generated from the neural precursors, and gives an overview of some of the complex cellular migration processes involved in the construction of the brain. The number of cells generated in the developing nervous system is likely regulated at several levels. In some cases, the production of neurons or glia may be regulated by an intrinsic limit in the number of progenitor cell divisions. The level of proliferation and ultimately the number of cells generated can also be controlled by extracellular signals, acting as mitogens, promoting progenitor cells to reenter the cell cycle or alternatively as mitotic inhibitors that induce progenitor cells to exit from the cell cycle. However, as we will see in Chapter 7, the number of neurons and glia in the mature nervous system is a function not only of cell proliferation, but also of cell death.

As we saw in Chapter 1, the nervous system of *C. elegans* (as well as the rest of the animal) is derived from a highly stereotyped pattern of cell divisions. Therefore, in these animals, the lineages of the cells directly predict their numbers. The regulation of these cell divisions appears to depend less on interactions with surrounding cells than is the case in vertebrates. The lineages of the *C. elegans* progenitor cells also predict the particular types of neurons that are generated from a particular precursor, and it appears that the information to define a given type of cell resides largely in factors derived directly from the precursors. The same is true for the neuroblasts that produce the *Drosophila* central nervous system: the production of neurons from the neuroblasts is highly stereotypic. The neuroblasts of the insect CNS delaminate from the ventral–lateral ectoderm neurogenic region in successive waves (see Chapter 1). In *Drosophila*, about 25 neuroblasts delaminate in each segment, and they are organized in four columns and six rows (Doe and Smouse, 1990). Once the neuroblast segregates from the ectoderm, it undergoes several asymmetric divisions, giving rise to approximately five smaller ganglion mother cells. Each ganglion mother cell then divides to generate a pair of neurons. These neurons make up the segmental ganglia of the ventral nerve cord and have stereotypic numbers and types of neurons.

In the vertebrate, the situation gets considerably more complex. The neural tube of most vertebrates is initially a single layer thick. As neurogenesis proceeds, the progenitor cells undergo a large number of cell divisions to produce a much thicker tube. A section through the developing spinal cord is shown in **Figure 3.1A**, and an example of a progenitor cell is shown as a schematic in **Figure 3.1B** and in the actual neural tube in **Figure 3.1C**, labeled with a fluorescent protein to visualize the cell as it progresses through a cell division. At this stage of development, almost all the cells in the neural tube resemble those shown in Figure 3.1B,C, with a simple bipolar shape. They extend one process to the central canal of the neural tube (named the ventricular surface because it is continuous with the ventricles of the brain) and they extend their other process to the outer surface of the neural tube. If one were just to look at the nuclei of the neural tube at this stage, there would appear to be many cell layers, and at first, the early neurohistologists thought this was the case. However, in the early 1900s it was recognized that the cells of the neural tube move their nuclei from the inside of the neural tube to the outside during each cell cycle. This movement can be directly observed using time lapse recording of cells labeled with fluorescent proteins (Figure 3.1C). This constant nuclear movement is termed interkinetic nuclear migration. In this process, the nuclei move to the inner, ventricular surface moment just before mitosis, and divide into two daughter cells; then the nuclei of these daughter cells move away from this surface during S-phase; but wherever they are just before the next mitosis, they rapidly move back to the ventricular surface to complete division (Norden et al., 2009). In the developing chick spinal cord, this process can take as little as six hours (Figure 3.1C). Although the function of interkinetic nuclear migration is unknown, it may be necessary for the

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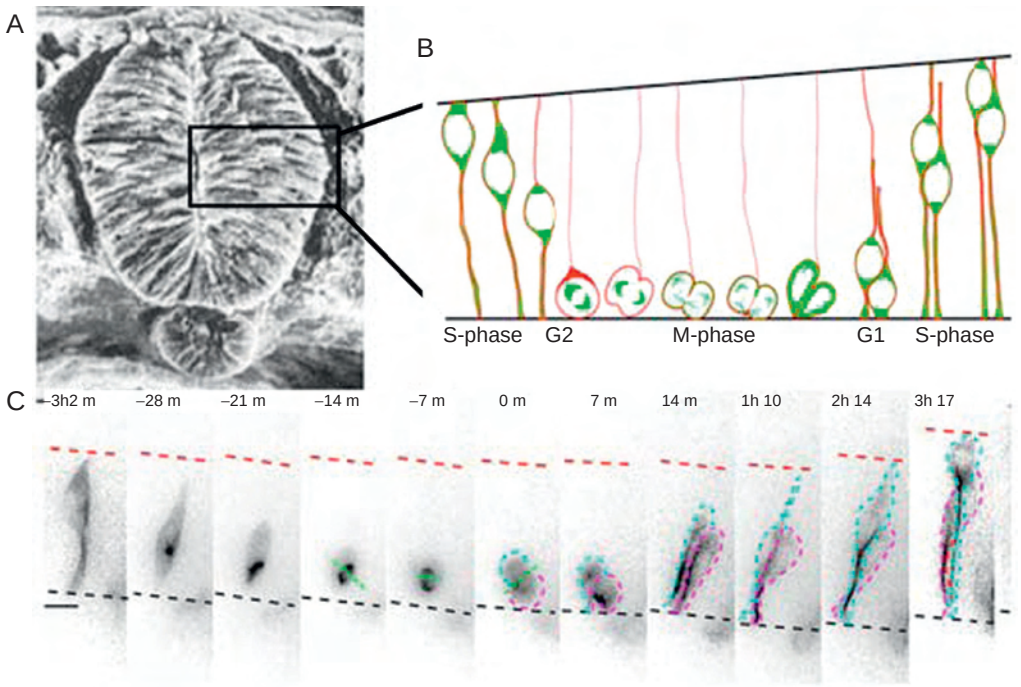

Fig. 3.1 The neural tube contains the progenitors of all the neurons and glia in the mature brain and spinal cord. A. These progenitors have a simple bipolar morphology. B. The nuclei of the progenitor cells undergo an "in and out" movement (shown here as an "up and down" movement) as they progress through the cell cycle. The cells move to the inside of the neural tube in the G2 phase of the cycle, go through the M-phase at the inner surface (also called the ventricular surface) and then move out again during S-phase. C. An actual time lapse recording showing a fluorescent labeled progenitor in the embryonic chick spinal cord undergo a mitotic division in a little over six hours. The M-phase lasts only about 20 minutes, while the S-phase can last much longer. *(A, Courtesy of Kathryn Tosney; B,C, Modified from Wilcock et al., 2007)*

progenitor cells to receive specific signals at different times in the cell cycle. On the other hand, Norden et al. (2009) propose that the migration to the ventricular surface prior to the mitotic division may be what is critical, and that the nuclei move away during S-phase to allow other nuclei access to that surface for their divisions. They compare this to people at a crowded pub, jostling around the room most of the time, but quickly returning to the bar for a refill.

The cells of the ventricular zone are the precursors of the differentiated neurons and glia of the central nervous system. In most other areas of the developing neural tube, once neurons and glia are generated by the progenitor cells, the neurons and glia migrate away from the ventricular surface to continue their differentiation elsewhere, and much more will be said of this process. Several methods have been developed to track the daughter cells of the progenitors once they leave the ventricular zone. One of the best ways to track the progeny of a progenitor is using a retrovirus that permanently marks all the daughter cells (**Figure 3.2**). The retroviral labeling technique takes advantage of the fact that retroviruses will only successfully infect and integrate their genes into cells that are going through the cell cycle. The genome of these viruses can be modified to contain genes that code for proteins not normally present in the nervous system but can be detected easily, such as green fluorescent protein (GFP). Once the virus infects a cell, and the viral genome is integrated into the cell's DNA, the viral genes are inherited in all the daughter cells of the originally infected cell. Another important feature of this technique is that the virus is typically modified so that it is incapable of making more virus in the infected cells and spreading the infection to other cells. This means that only the daughter cells of the *originally infected* progenitors will express the viral genes. If a retrovirus that contains DNA coding for GFP is then injected into the developing brain and infects some of the proliferating progenitor cells, the progeny of the infected cells will still express the GFP gene even in adult animals. This technique was used in the developing neural tube, and the distribution of clones has been analyzed shortly after injections as well as in the more mature spinal cord (Figure 3.2). When the neural tube was analyzed one day after the retroviral infection, a few progenitors expressed the reporter gene, and their progeny were clustered together, since they had not yet had time to migrate or differentiate; however, if the embryos were allowed to develop several more days to allow the spinal cord to mature, then the cells that express the reporter gene were typically more dispersed.v These cells could be shown to have developed from a single progenitor, and are therefore considered a "clone" and the technique is sometimes called "clonal analysis." In the case of the chick spinal cord, many of the clones contain both motoneurons and glial cells in the white matter. In the example shown in Figure 3.2, both astrocytes and oligodendrocytes are derived from the same progenitor cell that gave rise to the motoneurons (Leber et al., 1990). The clonal analysis method therefore not only allows one to track the cells as they migrate to other regions of the nervous system, but it also shows that the progenitors can make neurons and both types of macroglia found in the brain, the astrocytes and the oligodendrocytes. As a result, these progenitors are sometimes referred to as "multipotent progenitors," or at times "neural stem cells." (We prefer the former term, because the

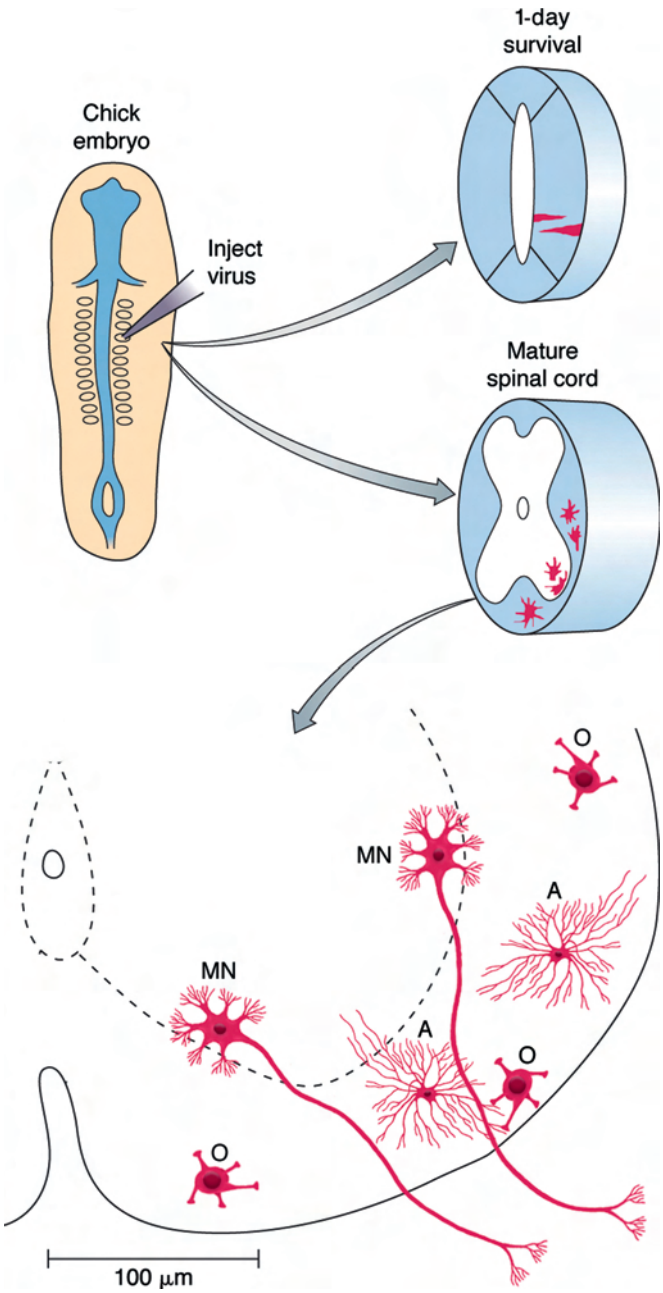

Fig. 3.2 Clonal analysis of progenitor cells in the chick neural tube. Injections of a retrovirus with a reporter gene are made into the chick embryo neural tube. After either short or long postinjection survival periods, the spinal cord is sectioned and analyzed for the labeled progeny of the few infected progenitor cells. In the case shown, a single progenitor cell has been infected at this level of the spinal cord, and it has gone through a single-cell division to give rise to two daughter cells after one day. If the embryo is allowed to survive to a point where the spinal cord is relatively mature, and neurons and glial cells can be identified, the labeled cells can be assigned to specific cell classes. In the case shown, the progeny of the infected cell include motoneurons (MN), astrocytes (A), and oligodendrocytes (O). *(Modified from Leber et al., 1990)*

term "stem cell" implies a self-renewal property that is difficult to assess. We will revisit this issue at the end of the chapter in the discussion of adult neurogenesis.)

In addition to the retroviral method for tracking the progeny of progenitors, a more classic method uses detectable analogs to the nucleotide, thymidine, to follow all cells generated at a particular stage of development. This method is called "^{3}H-thymidine birthdating." This technique, pioneered by Richard Sidman (1961), works as follows: While the progenitor cells are actively dividing, they are synthesizing DNA and incorporate thymidine into the new strand. If an animal is injected with an isotopically labeled form of thymidine (eg. ^{3}H-thymidine), this labeled form of thymidine gets incorporated into the DNA of the mitotic cells. ^{3}H-thymidine is incorporated into DNA during replication like ordinary thymidine, but because it is radioactive, it can be traced with a technique called autoradiography. Typically, a single injection of the ^{3}H-thymidine is given and therefore it is available for only a few hours. The progenitor cells that were in the S-phase of the cell cycle at the time the injection was made will be labeled with the ^{3}H-thymidine; however, if they continue to proliferate over many days, the labeled DNA will be diluted over time. By contrast, those cells that withdraw from the cycle and become postmitotic soon after the ^{3}H-thymidine was administered will remain heavily labeled with the radioactive nucleotide. Thus, the postmitotic neurons generated, or "born," within a day after the ^{3}H-thymidine injection will have heavily labeled nuclei, and neurons generated later in development will be more lightly labeled. Unlabeled cells are those that withdrew from the cell cycle before the ^{3}H-thymidine injection. More recently, ^{3}H-thymidine labeling has been replaced by bromo-deoxyuridine (BrdU), since this thymidine analog is also incorporated by S-phase cells and can be detected using an antibody and immunofluorescence, rather than the more complicated autoradiography technique.

The ^{3}H-thymidine birthdating has been used extensively to track the migration and birthdates of the different neuronal and glial populations in the nervous system. These studies revealed that the process of neurogenesis is remarkably well-ordered. In many areas of the developing brain there are spatial and temporal gradients of neuron production. In general, there are well-conserved and orderly sequences of generation of different types of neurons and glia. For example, in the cerebral cortex, the neurons are arranged in layers or lamina. If mice are labeled with ^{3}H-thymidine at successively later stages in their development (**Figure 3.3A**), the labeled cohort of newborn neurons forms a layer more superficial than the previous one. This "inside-out" pattern of neurogenesis is found in the cerebral cortex of all mammals, from mice to monkeys (**Figure 3.3B**), and presumably people as well.

Several additional generalizations can also be derived from the large number of thymidine birthdating studies that have been carried out in the different regions of the vertebrate CNS. As noted above, in many areas of the developing CNS, distinct types of neurons originate in a fairly invariant timetable. Often, the entire population of one type of neuron, like the spinal motoneurons, becomes postmitotic within a relatively short period of development. In general, large neurons are generated before small neurons in the same region. For example, pyramidal cells become postmitotic before granule cells in the hippocampus, cerebral cortex, and olfactory bulb; and in the cerebellum, Purkinje cells are generated prior to granule cells (Jacobson, 1977). Interestingly, it appears that the patterns of neuronal generation are also consistent with the hypothesis that phylogenetically older parts of the brain develop before the more recently evolved structures.

The picture of neurogenesis that has emerged from the thymidine birthdating studies and the retroviral lineage studies has led to many questions about the process: What controls the number of neurons and glia produced by the progenitors? How does a progenitor "decide" whether to make a neuron or a glial cell?

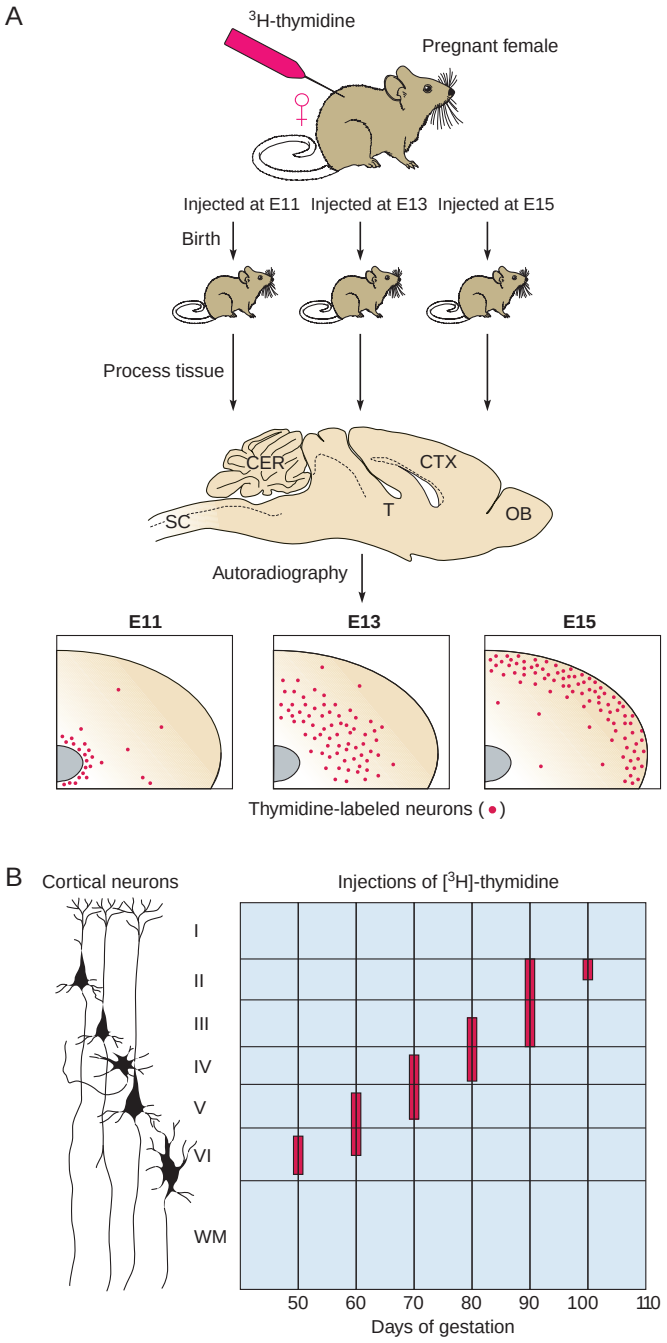

Fig. 3.3 A. Birthdating studies demonstrate the inside-out pattern of cerebral cortical histogenesis. Pregnant female rats are given injections of ^{3}H-thymidine at progressively later stages of gestation. When the pups are born, they are allowed to survive to maturity, and then their brains are processed to reveal the labeled cells. Neurons that have become postmitotic on embryonic day 11 are found primarily in the subplate (now in the subcortical white matter), while neurons "born" on day E13 are found in deep cortical layers, that is, V and VI, and neurons generated on E15 are found in more superficial cortical layers, that is, IV, III, and II. The most superficial layer, layer I, contains only the remnants of the preplate neurons (not shown). *(Modified from Angevine and Sidman, 1961)* B. Similar thymidine birthdating results in the monkey show this pattern more clearly than in the mouse due to the longer period of gestation.

What controls the migration of the cells from the ventricular zone to their ultimate location in the brain? The next sections will highlight what is known about these questions.

WHAT DETERMINES THE NUMBER OF CELLS PRODUCED BY THE PROGENITORS?

The thymidine birthdating studies and the retroviral lineage tracing studies described above provided a wealth of information about the migrations and cell types generated by the progenitors; however, they also provided information about how cell numbers are regulated during development. For example, with the thymidine method, it is possible to determine the length of the cell cycle, and it was shown that overall length of the cell cycle increases progressively during embryogenesis. Progenitor cells from the chick brain, for example, have an overall cell cycle time of 8 hours on embryonic day 3, but this increases to 15 hours by embryonic day 6. A similar increase in cell-cycle period occurs in the mammal, as rat cortical progenitor cells increase their cell-cycle time from 11 hours on embryonic day 12 to 19 hours at embryonic day 18. The second generality that can be made is that the increase in the cell-cycle period is largely due to increases in the G1 phase. As shown in **Figure 3.4**, the M and G2 phases of the cell cycle change little from embryonic

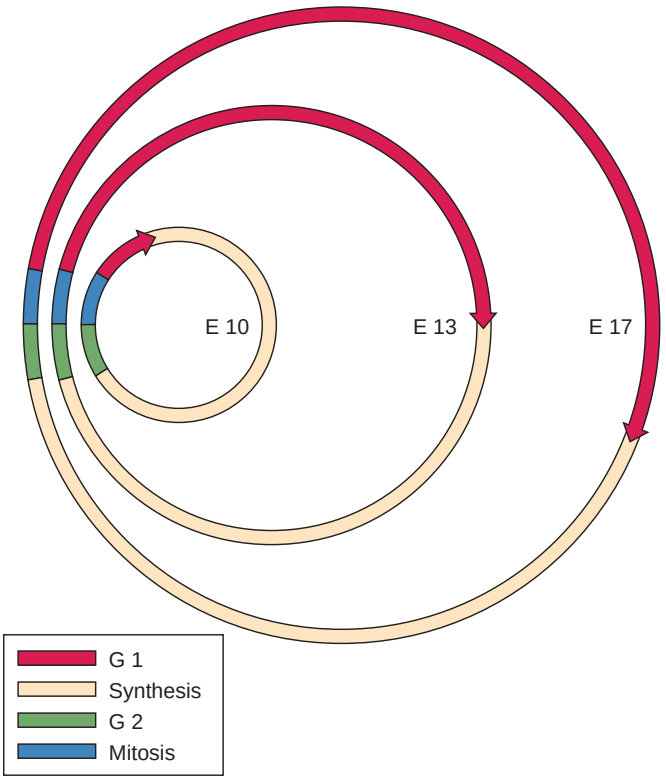

Fig. 3.4 The overall length of the progenitor cell cycle increases during embryogenesis. The cell cycles of progenitor cells from the mouse cerebral cortex are plotted as circles of increasing size from E10 to E17. The increase in the cell-cycle period is largely due to an increase in the G1 phase, which nearly triples in length (shown in red).

day 10 to embryonic day 19 in mouse cerebral cortex progenitor cells; however, the G1 phase nearly triples in length. The lengthening of the G1 period likely reflects some regulatory process that restricts or slows reentry of the progenitor cells into the S-phase from G1, consistent with the idea that a limiting supply of growth factor controls this step (see next section).

Labeling individual progenitor cells with retroviruses at different stages of brain development has shown directly that the number of progeny generated by a ventricular zone cell declines over the period of neurogenesis. For example, retroviral infections of the progenitor cells in the early embryonic brain result in very large clones of labeled cells, but retroviral infections of progenitor cells in the brains of late staged embryos gives much smaller clones. Simply counting the rate of overall expansion of the nervous system over time has also led to insight into the process of neurogenesis. In the early embryonic cerebral cortex, for example, the number of cells doubles each day. Since it takes approximately 12 hours for a progenitor cell to generate two daughters, more than half of the progeny must continue to divide; that is, many of the cell divisions must produce two mitotically active daughters. During this early "expansion phase" of the progenitor cells, most of the cell divisions are symmetric, generating two additional progenitor cells (**Figure 3.5**). As development proceeds, and the cell-cycle time becomes progressively longer, the number of new cells generated per day declines. Fewer cell divisions are symmetric and result in two progenitor cells at later stages of development, compared to the early stages of embryogenesis. Instead, in the later stages of neurogenesis, a greater proportion of the progenitor cells differentiate into neurons and glia. By the end of neurogenesis, nearly all of the cells leave the cell cycle, and very few remain to generate new neurons. From these results then it seems that the answer to the question of how cell numbers are regulated during development of the brain might be divisible into two subquestions: (1) What factors account for the gradual lengthening of the cell cycle during development? (2) What factors control the shift from symmetric "expansion phase" cell divisions of the progenitors to their neurogenic, asymmetric divisions? As we will see below, regulation of these aspects of the process can have profound effects on the total number of cells produced by the progenitors.

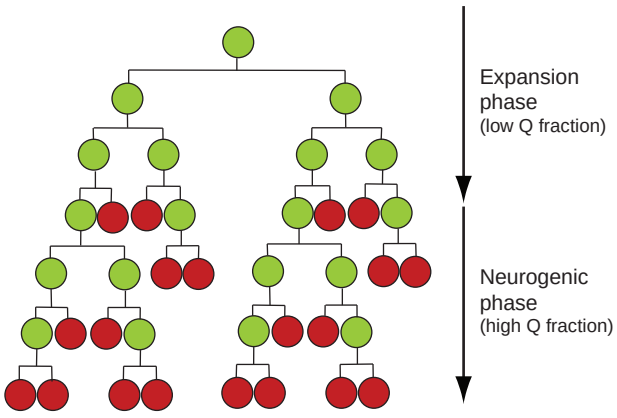

Fig. 3.5 Stages of neurogenesis. Early in development, the progenitor cells (green) divide symmetrically to produce two more progenitor cells. During this expansion phase of histogenesis, the progenitor population expands rapidly. In the middle phase of histogenesis, the progenitor cells divide asymmetrically to produce another progenitor and a postmitotic neuron (red) (sometimes call the Q or quit fraction because they do not reenter the mitotic cycle). In this neurogenic phase, the progenitor pool is stable, but not expanding. However, neurons are being produced and so the total cell number is increasing. At the end of histogenesis, the progenitors produce two postmitotic progeny (either neurons or glia) and the progenitor pool is depleted.

The answers to these questions came in part from the identification of the molecular machinery that powers the mitotic cell cycle. Many of the same molecular mechanisms that control the proliferation of progenitors in the nervous system are also important for the control of cell division in other tissues (**Figure 3.6**). Through the analysis of mutations in yeast cells that disrupt normal cell cycle, a number of the components of the molecular machinery controlling cell cycle have been identified. An intricate sequence of protein interactions controls and coordinates the progress of a cell through the stages of cell replication. This molecular mechanism has been conserved over the millions of years of evolution from the simplest eukaryotic cells, like yeast, to more complex animals and plants. Key components of the cell cycle control process are called cyclins, a group of proteins that show dramatic changes in their expression levels that correlate with specific stages of the cell cycle. The association of cyclins with another class of proteins, called the cyclin-dependent kinases (Cdk), causes the activation of these kinases and the subsequent phosphorylation of substrate proteins necessary for progression to the next phase of the cell cycle. Different cyclin/Cdk pairs are required at different stages of the cell cycle. For example, the binding of cyclinB to Cdc2 forms an active complex that causes a cell to progress through the M-phase of the cycle, while the association of cyclinD and Cdk4 or Cdk6 regulates a critical step in the progression from G1- to S-phase.

One of the critical steps in the control of the cell cycle is at the transition from the G1 stage to the S-phase, and as noted above, cyclinD is an important regulator of this step (Figure 3.6). The cyclinD/Cdk4 complex causes cells to enter S-phase by phosphorylating a protein called retinoblastoma or Rb. This phosphorylation causes the Rb protein to release another transcription factor, E2F, and allows the E2F protein to activate many genes that push the cell into S-phase. The Rb protein received its name from a childhood tumor of the retina, retinoblastoma, since defects in this gene cause uncontrolled retinal progenitor proliferation. In fact, the Rb gene was the first of a class of genes called tumor suppressors to be identified. Children who inherit a mutant copy of the *Rb* gene develop retinoblastoma when the second allele of this gene is mutated in a progenitor cell in the retina. E2F is then free to activate the genes that cause the progenitor to progress through the cell cycle, and there is no active Rb around to stop the process. Thus, the regulation of progenitor proliferation is critical both for making a normal retina and for preventing the uncontrolled cell proliferation that leads to cancer. There are also proteins that inhibit the cell cycle. Two of these, $p27^{kip}$ and p21, are also expressed in the nervous system, and they are expressed in the final mitotic cycle of a progenitor, causing it to exit the cell cycle and differentiate into neurons or glia. The $p27^{kip}$ and p21 gene products are therefore called CdkIs (for Cdk inhibitors).

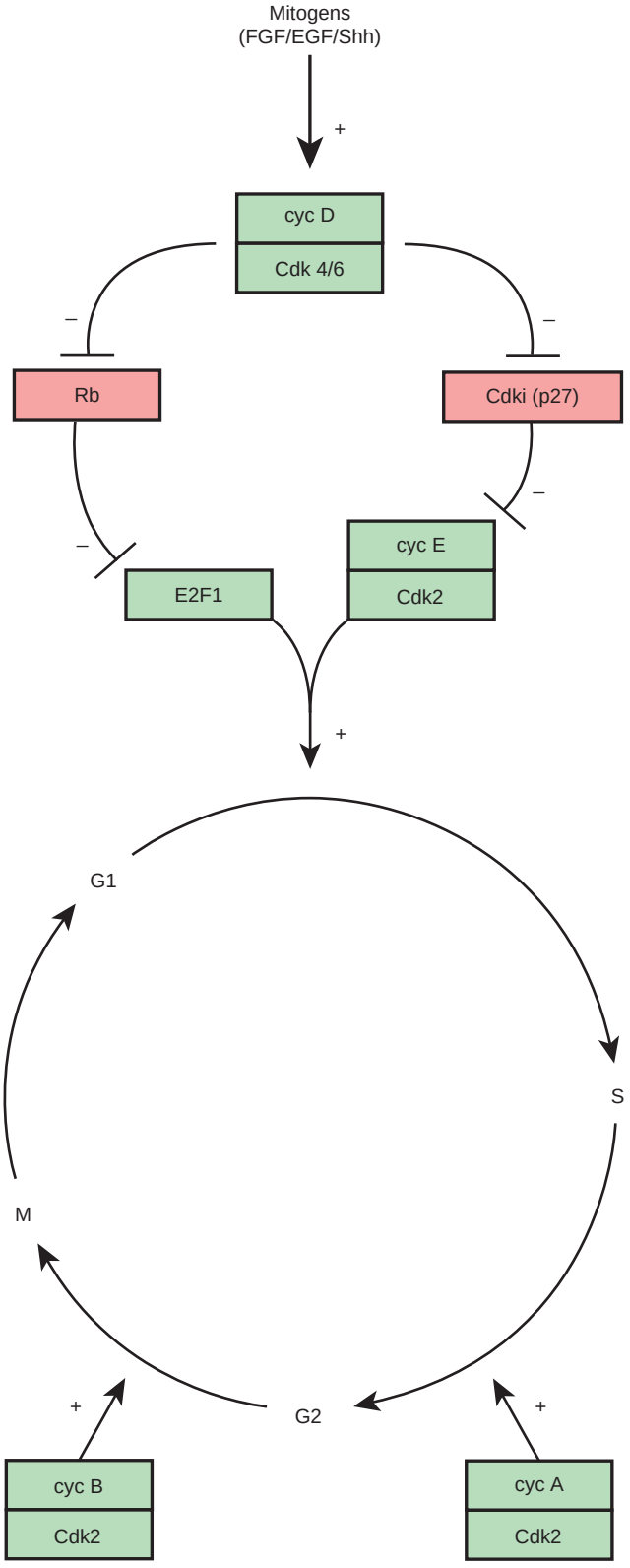

Fig. 3.6 Basic molecular mechanisms of the mitotic cell cycle. Molecules that promote cell proliferation are shown in green and those that inhibit the cell cycle are shown in red. The entry of a cell into the S-phase is one of the key check points on mitosis. E2F1 and cyclinE/Cdk2 complexes cause cells to enter S-phase. However, there are "brakes" on S-phase entry, the Rb protein and cyclin dependent kinase inhibitors, like $p27^{kip}$. Mitogens that stimulate cells to enter the cell cycle, like EGF and FGF, stimulate cyclinD expression or stabilization, which then inhibits the "brakes" on the system and promotes S-phase.

Caviness and his colleagues (Caviness et al., 2003) have developed quantitative models to investigate the role that cell cycle regulators can have in controlling cell number during neurogenesis in cerebral cortex. They have found that $p27^{kip}$ plays a key role in this process. Total cell output from the mitotic divisions of the cortical progenitors can be expressed as the P (or progenitor fraction) + Q (or the quit fraction). The Q fraction is composed of postmitotic neurons, and so the daughters of a progenitor division that choose a neuronal fate no longer contribute to the production of additional neurons. In the early embryonic cerebral cortex, the percentage of the total in the Q fraction is relatively small, and those cells in the P fraction continue to divide and produce more cells. However, as development proceeds, the percentage of cells in the Q fraction increases and the overall growth rate of the cortex declines. If $p27^{kip}$ is experimentally reduced, by knocking out the gene in mice, a smaller percentage of cells enter the Q fraction, and the resulting cortex is noticeably thicker (**Figure 3.7**). On the other hand, the converse experiment of

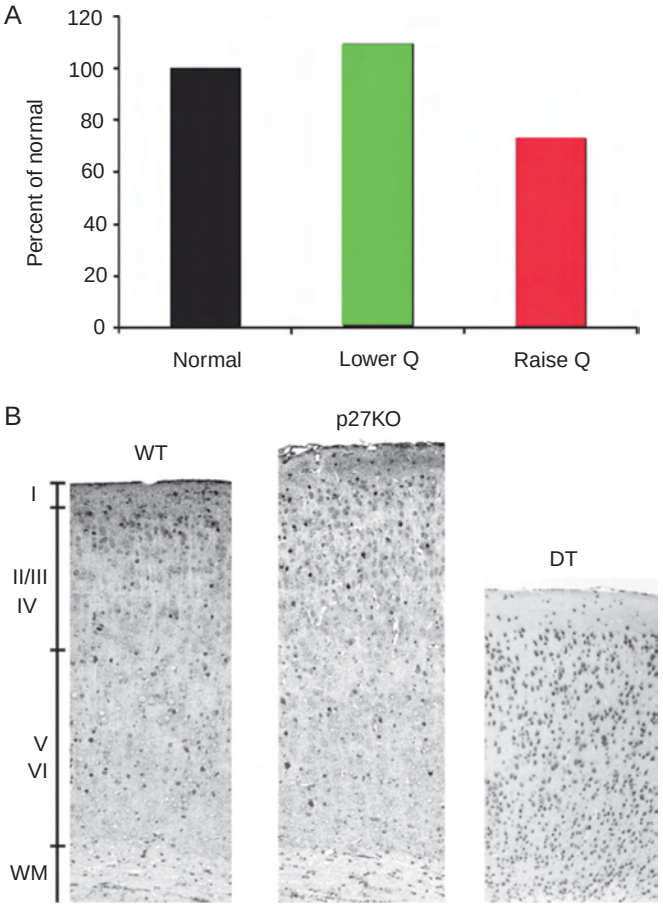

Fig. 3.7 Cerebral cortex growth and cell cycle regulators. A. The total number of neurons produced in the cerebral cortex is a function of the number of cell divisions of the progenitors that produce more progenitors (P) and the divisions that produce neurons (the "quit" or Q fraction). Mathematical modeling of this process shows that a slightly lower Q fraction produces an increase in the number of neurons in the cortex (green), while a higher Q fraction depletes the progenitor pool earlier in development and results in a smaller total cell number (red). B. Actual cortical thickness of mice in which $p27^{kip}$ was genetically deleted (KO), resulting in a lower Q fraction and a larger brain. By contrast, if the Q fraction is experimentally raised in mice, the cortex is markedly thinner. *(Modified from Caviness et al., 2003)*

over-expressing $p27^{kip}$ leads to a greater percentage of cells in the Q fraction, and a markedly thinner cortex. Therefore, the level of the CdkI $p27^{kip}$ modulates the probability that a cell will enter the Q fraction. Since different cortical layers are generated at different times during development (see below), the level of $p27^{kip}$ expression also affects the relative numbers of cortical cells in the various layers.

The studies of cyclins and their regulators have indeed revealed part of the answer to the question that began this section; however, we have really just pushed the question back a step and you may be wondering: "what regulates the cyclins?" Once again, the progenitors use many of the same regulatory factors as other tissues in the body. In many tissues in the body, secreted signaling factors have been identified that stimulate or inhibit the progress of mitotically active cells through the cell cycle. The signals that stimulate the proliferation of the mitotic cells are called growth factors or mitogens and were named for the tissue or cell type where they were first found to have mitogenic effects. For example, fibroblast growth factor (FGF) was first found to promote the proliferation of fibroblasts in cell cultures, whereas epidermal growth factor (EGF) was discovered as a mitogen for epidermal cells in vitro. These growth factors most commonly act to control the progression from G1- to S-phase of the cell cycle, in part by controlling the level of expression of cell cycle regulator proteins like cyclinD1. One potential explanation for the gradual lengthening of the G1 phase of the cell cycle in the progenitor cells at later stages of development (above) is an increasing dependence on these mitogenic growth factors for progression through the cell cycle as development proceeds. The factors that have been shown to act as mitogens for the the progenitor cells of the vertebrate CNS are primarily those peptides that act on receptor tyrosine kinases, including FGFs, TGF-alpha, EGF, and insulin-like growth factors. However, there are many other types of signaling molecules that act on progenitor cells in the nervous system and also play a role in their proliferation. Sonic hedgehog and members of the Wnt protein family are examples of molecules that are involved in patterning the nervous system (reviewed in Chapter 2), but are also critical for the regulation of progenitor proliferation at later stages of brain development. Progenitor cells express receptors for the various mitogenic factors, and depending on their location and stage of development, they are more responsive to one mitogen or another. Mitogenic factors like EGF and FGF stimulate cell division by the upregulation of the S-phase cyclins (Figure 3.7), such as cyclinD. On the other hand, there are also signaling molecules that act as "stop signals" for proliferation, like TGF-beta. These work through surface receptors to upregulate expression of cell cycle inhibitors, like $p27^{kip}$. The progenitors must integrate these signals to determine whether they progress to the next S-phase and in this way the extracellular signals are connected with the intrinsic cell cycle regulation machinery to allow for the correct cell numbers in each region of the brain.

In this section we have seen that the regulation of the numbers of neurons and glia in the developing brain is influenced by factors that cause the gradual lengthening of the cell cycle during development and factors that control the shift from symmetric "expansion phase" cell divisions of the progenitors to their neurogenic, asymmetric divisions. In fact, there is some evidence that the two processes might be intimately connected; Calegari et al. (2005) proposed that, as the cell cycle length gets progressively longer over the period of neurogenesis, this *causes* the progenitors to switch from generating additional progenitors, to the generation of neurons, though the mechanisms for this connection are not yet known. In the next section, we will discuss the mechanisms that control the developmental decision of the progenitor to produce neurons, glia or both. This process also appears to be tied to the developmental stage of the cell.

THE GENERATION OF NEURONS AND GLIA

Retroviral lineage studies have shown that, for many regions of the nervous system, neurons, astrocytes, and oligodendrocytes can arise from a single infected progenitor cell. In addition, these studies also showed that the ratio of the different types of cells produced by a progenitor is quite variable. One multipotent progenitor might produce only a few neurons, but many astrocytes, while another might generate mostly neurons. Therefore, the lineages of multipotent progenitors are considered to be "indeterminant" in the vertebrate CNS. Early in development, most of the progenitors are multipotent, but in some regions of the brain, there are committed progenitors that produce only neurons or only glia. What controls the relative number of these different types of cells made from the multipotent progenitors? What distinguishes the multipotent progenitors from the committed progenitors? Tracking the potency of single progenitor cells in vitro has shed some light on these questions. **Figure 3.8** shows the lineages of four different progenitor cells that were isolated from the developing brain, and then maintained in tissue culture over several days; the cell divisions of each progenitor cell were followed by direct observation, and the cell types they generated were confirmed at the end of the culture period by labeling each cell type with an antibody that uniquely recognizes each type of cell (Figure 3.8A). The lineage diagram (Figure 3.8 B) shows that each of the progenitor cells produces a different number of neurons and glia, and so the lineages are indeterminant in vitro as they are in vivo. It can also be seen from the figure that at the beginning of the observation period, two of the four progenitors that were followed over time produced neurons, astrocytes, and oligodendrocytes, while one generated only oligodendrocytes and one produced neurons and oligodendrocytes. Looking more closely, sometimes two different cell types were produced by the last cell division, but more commonly, the multipotent progenitor cells eventually produce bipotent and then unipotent progenitor cells. For example, cell number 2 is tri-potent at the start, but after one generation produces two bipotent progenitor cells, one of which makes neurons and oligodendrocytes and the other makes neurons and astrocytes. These data suggest that the potential of progenitor cells becomes progressively restricted over time and that unipotent progenitor cells are derived from multipotent progenitors.

What controls the progressive restriction in potential of the progenitor cells? Cell culture studies indicate that both extracellular signaling factors, like those that control cell proliferation of the progenitor and intrinsic processes within the cells, play important roles in regulating the potential of the progenitor cells to either a neuronal or glial lineage. In cell cultures, one can add defined factors and assay the effects on the production of either neurons or glia from the progenitor cells. These kinds of studies have led to some general

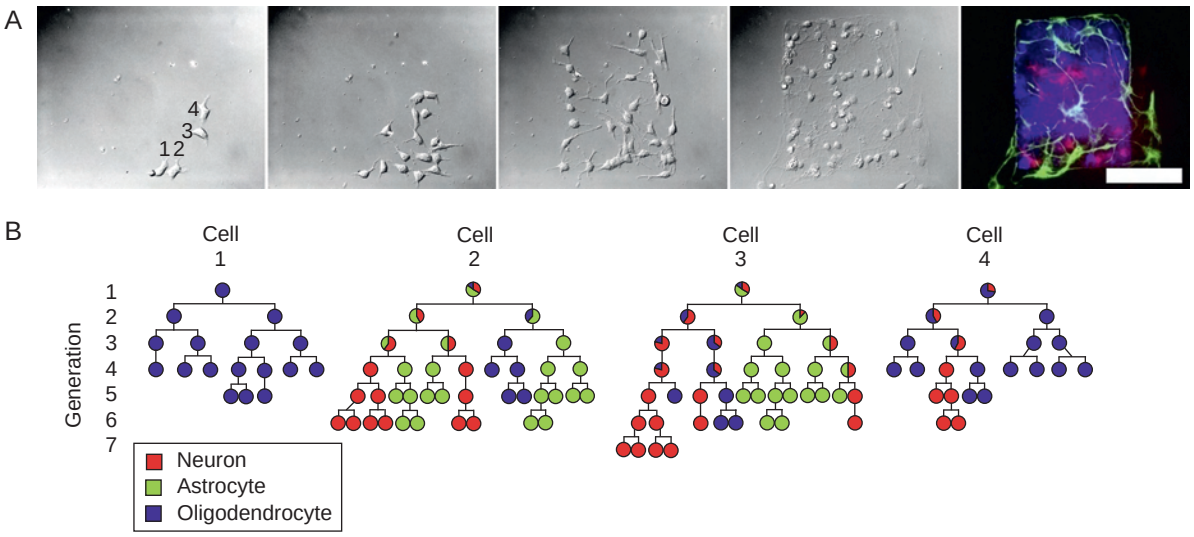

Fig. 3.8 The proliferation of neural progenitor cells can be studied in vitro. A. Progenitor cells from the developing CNS can be studied in cell culture by dissociating them into single cells, diluting them to only a few cells in each well of a tissue culture dish, and then examining them daily for increases in their numbers. B. These micrographs, taken daily, document the proliferation of progenitors. Labeling the culture with antibodies against cell-specific protein shows that several of the new cells have developed into neurons, while others express antigenic markers of either oligodendrocytes or astrocytes. (*A, B, Modified from Ravin et al., 2008)*

principles. In general, the addition of fibroblast growth factors (FGFs) to culture of neural progenitors causes them to increase their differentiation into neurons. By contrast, when cultures of progenitors are cultured in the presence of epidermal growth factor (EGF), ciliary neuronotrophic factor (CNTF), or bone morphogenic proteins (BMPs), the cells are more likely to develop as astrocytes. Still other factors, like PDGF (platelet-derived growth factor), promote oligodendroglial development when added to similar cultures (Raff et al., 1988). These pathways are summarized in **Figure 3.9**. While there are many exceptions to these generalizations, the effects of CNTF on astrocyte development have been particularly well worked out (Bonni et al., 1997; Rajan and McKay, 1998). The activation of the CNTF receptor leads to phosphorylation of a downstream signaling molecule, STAT3. The active STAT3 goes directly into the nucleus, binds to the promoter and activates the glial specific genes GFAP and S100. Thus, this provides a direct transcriptional connection between the signaling molecule and a glial-specific gene. BMP synergizes with the CNTF to give an even more robust response. The early progenitors are relatively unresponsive to CNTF signaling, and hence few glia are produced early in development. However, as embryogenesis continues, glia begin to be generated. What accounts for the increased response of these late progenitors to the gliogenic signal CNTF? The responsiveness of the cell to the CNTF signal is an intrinsic property of the progenitor that changes over development. The DNA in the promoter of GFAP is methylated in the early progenitors, so that the STAT3 cannot bind there and activate GFAP expression (Takizawa et al., 2001; Fan et al., 2005). A similar block in access is present in the promoters of other glial genes, and hence, early progenitors are blocked from producing astrocytes. This interplay between signaling factors in the local environment of the progenitors, along with intrinsic properties of the cells, allows for the developmental program to respond to the surrounding cells.

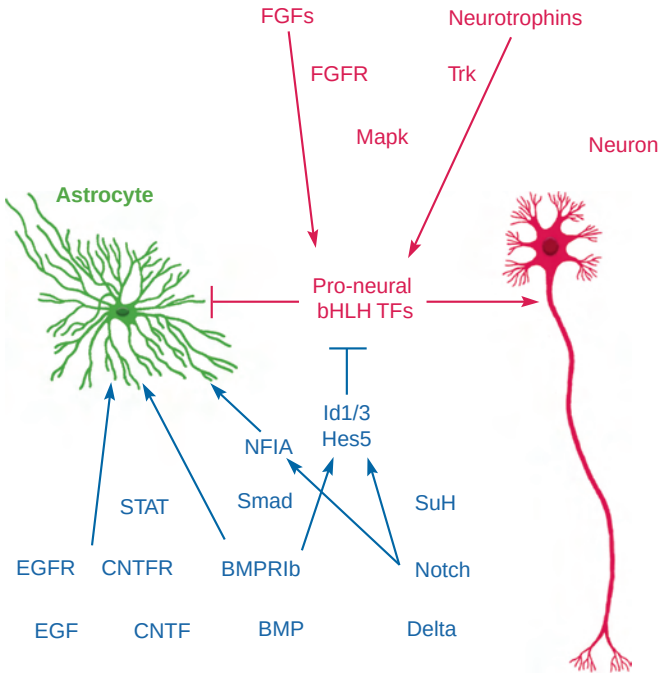

Fig. 3.9 Various mitogenic factors control proliferation of the different types of progenitors in the nervous system. Neurogenesis and gliogenesis are regulated by many growth factors, and these are summarized in the figure. *FGF2* and *Neurotrophin3* promote progenitor cells isolated from the brain to develop primarily as neurons, likely through the increase in expression of proneural *bHLH* genes, such as *NeuroD1*, EGF, and CNTF, which cause the progenitor cells to develop as astrocytes, and at least for CNTF this is known to work through the activation of the STAT transcription factor, which binds to the promoter of the glial-specific gene, GFAP. BMPs can synergize with CNTF to promote glial development, partly through the STAT pathway and partly through a direct inhibition of the proneural genes via the *Hes* pathway. Notch activation drives expression of the astrocyte-specific transcription factor, NFIA, and at the same time also activates the *Hes* pathway to promote gliogenesis and inhibit neurogenesis.

Another important pathway that regulates the production of neurons and glia is the Notch pathway. As we saw in the previous chapters, the Notch signaling pathway and the proneural transcription factors are important in the early stages of nervous system formation. These genes also play critical roles in the process of neurogenesis (Bertrand et al., 2002). The components of the Notch pathway and the proneural transcription factors are expressed in the progenitors and the differentiating neurons. The progenitor cells express several proneural transcription factors, including Mash1, Neurogenin, and Olig1/2. These proneural transcription factors are important for maintaining the progenitors by activating the expression of the Notch ligands, Dll1/3 (Kageyama et al., 2008). The Notch ligands, in turn, activate the Notch receptors on the progenitor cells, and activate the expression of the Hes genes, Hes1/5, and related factors. The Hes genes, and the Notch receptor itself, are necessary for the maintenance of the progenitor state in the cells. Blocking the Notch receptor, either genetically or with specific inhibitors, leads to the premature differentiation of these cells into neurons (Nelson et al., 2007). Over-expression of activated Notch causes the opposite: the progenitor cells fail to differentiate into neurons, and either remain progenitors, or become glia (see below). If all the progenitors have approximately equal levels of Neurogenin, Dll, and Hes1, the progenitor pool is maintained; however, if one of the daughter cells from a mitotic division expresses a higher level of Neurogenin than the other, it will also express more Dll; this will activate Notch in the sister progenitor cell at a higher level, and lower its level of Dll, leaving the cell with more Neurogenin free to differentiate as a neuron. Creating even a small bias in the two daughter cells in their expression of Neurogenin, its repressor, Hes1, or the activity of the Notch receptor, would lead to the amplification of the difference because of this feedback between the two cells. In this way, the decision of one cell to become a neuron, while the surrounding cells remain as progenitors is similar to the developmental decision in the *Drosophila* of one cell from the proneural cluster to become the neuroblast while the surrounding cells remain as epidermal cells (**Figure 3.10**).

In addition to the basic feedback loop between cells created by the Notch pathway, Kageyama's group found that Hes1 protein represses its own transcription, and this leads to a simple feedback loop that causes the protein levels to oscillate within each progenitor cell every 2–3 hours (Shimojo et al., 2008). This Hes1 oscillation within each progenitor cell causes a counter-oscillation of the Neurogenin and Dll in each cell. Now when two progenitors, each with their own Hes1/Neurogenin cycle are brought in contact, they should cycle in opposite phase to one another. A second oscillation in the progenitor cell expression of Notch pathway activity also occurs with the mitotic cell cycle. Hes1 levels are higher when the cell is in the S-phase and lower as the cells enter the M-phase and G1 phase of the cell cycle (near the ventricular surface). The inhibition of Notch signaling at the M-phase appears to

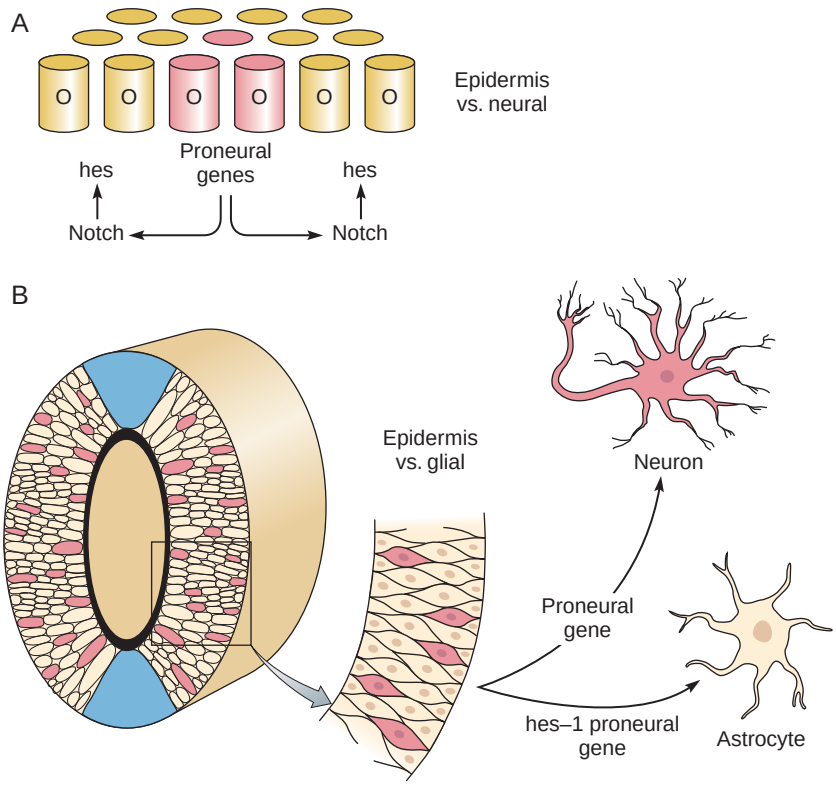

Fig. 3.10 The proneural genes and the Notch pathway regulate neurogenesis. A. As described in Chapter 1, the proneural genes are important in the initial segregation of neural tissue from the epidermis in both *Drosophila* and vertebrates and function through an inter-cellular feedback loop to amplify small biases between cells. Proneural transcription factors induce expression of Notch ligands (DL) which then activate the Notch receptor in adjacent cells (N) to drive *Hes1* expression, which inhibits the proneural genes. B. Within the progenitor population in the ventricular zone, all the cells express these genes at some level. Those cells that express higher levels differentiate as neurons (red).

be due to the release of the Notch pathway inhibitor ACBD3 from the Golgi as the cell divides (Zhou et al., 2007). How are the oscillations in Hes1 and Notch signaling related to neurogenesis? Although the molecular mechanisms underlying the oscillations in Notch signaling are known, it is not clear whether these are critical for the process of neurogenesis.

The Notch pathway is also critical for regulating gliogenesis. Over-expression of activators of this pathway in progenitors (e.g., Hes1, Hes5 or activated forms of the Notch receptor; Vetter and Moore, 2001) can either maintain the cells in the progenitor state or, in some cases, cause the cells to become glia, primarily astrocytes. Since astrocytic glia are frequently produced later than neurons in most areas of the nervous system, it could be that the activation of the Notch pathway simply prevents progenitors from differentiating until other signals that induce glial differentiation are produced. As we have seen above, signaling molecules like BMP and CNTF promote gliogenesis, through their activation of glial-specific genes. However, there is other evidence that over-activating Notch plays an instructive role in gliogenesis as well as this more permissive role. As noted above, the early progenitors are blocked from making astrocytes because the DNA in the promoters of glial-specific genes is methylated, inhibiting access of STAT3. Activated Notch induces the demethylation of the STAT3 binding site of the GFAP promoter, so that CNTF can better activate this pathway (Namihira et al., 2009). Along with the demethylation of the GFAP promoter, Notch activation also induces the expression of a glial promoting transcription factor: NFIA. NFIA is expressed by the late staged progenitors during the time when astrocytes are being generated (Deneen et al., 2006). NFIA is both necessary and sufficient for induction of astrocyte genes: knocking this gene out in mice leads to a reduction in astrogliogenesis, while over-expressing this gene leads to an increase in astrocyte production by the progenitors. At the same time the NFIA activates the gliogenesis program (Figure 3.9), it is also important in the repression of neurogenesis from the progenitors. The ability of NFIA to repress neurogenesis is mediated at least in part by Hes5, which as we saw in the previous section, is downstream of Notch signaling. This nicely ties together the findings that Notch and Hes5 can promote the glial fate.

In addition to astrocytes, the other type of macroglia in the central nervous system is the oligodendrocytes. What are the mechanisms that control their formation during neural development? As noted above, the lineage studies in vitro and in vivo showed that progenitor clones could contain neurons, astrocytes, and oligodendrocytes. This means that at least early in development, there are tripotent progenitors. It was observed several years ago, though, that the oligodendrocytes only arise from the ventral part of the spinal cord, and further studies found they only arise from the progenitors in a relatively small part of the ventral ventricular zone, called the pMN, since it is also the region that produces the motor neurons (MN). As we saw in the previous chapter, the signaling molecule Shh is critical for the determination of ventral fates in the spinal cord, and so is also necessary for the specification of this zone and the production of motor neurons (see Chapter 4). A search for the genes that are necessary for oligodendrocyte development led to the discovery of two transcription factors called Olig1 and Olig2. These transcription factors, are specifically expressed in the pMN zone, and when they are knocked out in mice, the oligodendrocytes fail to develop. In addition, over-expression of Olig1 or Olig2 can induce additional oligodendrocytes, and so it can be considered part of the transcriptional network that controls development of this cell type. However, the Olig1/2 knockout mice have an additional problem: the motor neurons don't develop either. These results would indicate that the progenitors in this region are the ones that are competent to make both neurons and oligodendrocytes. Birthdating studies and retroviral lineage studies have both shown that the motoneurons are produced prior to the oligodendrocytes. Taking these data together, it would appear that the same progenitors make both cell types, but they initially generate motoneurons and then switch to oligodendrocytes production. What accounts for the switch in cell type production by these cells? At the time the cells make motoneurons, they express the proneural gene, Neurog2 (Kessaris et al., 2001; Zhou et al., 2001). Neurog2 and Olig1/2 combine their activity to generate motoneurons. Later in development, however, the cells turn off Neurog2 and produce a very different type of transcription factor, Nkx2.2; Nkx2.2 is a repressor of motoneuron genes (see Chapter 4), but it does not repress Olig1/2 expression in this region. Now the progenitors that express both Olig1/2 and Nkx2.2 start making oligodendrocytes (**Figure 3.11**). This molecular switch enables progenitor cells to produce different types of progeny at different times in development. A progenitor cell from this region of the neural tube would initially make neurons, but after one or two generations, it would progress to a state where it was generating oligodendrocytes.

The foregoing two sections have shown one of the common themes in the process of neurogenesis: intrinsic changes in the progenitor cells over developmental time determine the cell's responsiveness to signaling factors produced by neighboring cells. The developmental dance between cell-intrinsic and cell-extrinsic regulation of cell division and neuron or glial production allows for great flexibility in the numbers and relative proportions of neurons and glia in the different regions of the brain and in different species. This theme continues in Chapter 4 as we consider the question of neuronal diversity.

CEREBRAL CORTEX HISTOGENESIS

The cerebral cortex has been particularly instructive in elucidating the principles of histogenesis in the developing brain. Histogenesis is the process by which architecturally organized regions of the brain, such as the six-layered cortex, can be understood in terms of the timing of neurogenesis. The human neocortex has been called the "most complicated object in the universe," and there is no doubt that this structure endows us with remarkable cognitive abilities. The six-layered neocortex (**Figure 3.12**) is a uniquely mammalian structure, and it reaches its most extensive elaboration in humans. There has been a dramatic increase in size during evolution; comparing the surface area of the mouse, macaque monkey and human cerebral cortex gives ratios of 1 to 100 to 1000, and this has been accompanied by an increase in the number of distinct, identifiable regions, based on the relative numbers of neurons in each layer. The layers are numbered from the most superficial, layer I, to the deepest, layer VI. While all regions of the neocortex have these six layers, there are variations in the relative numbers of neurons in the different cortical regions,

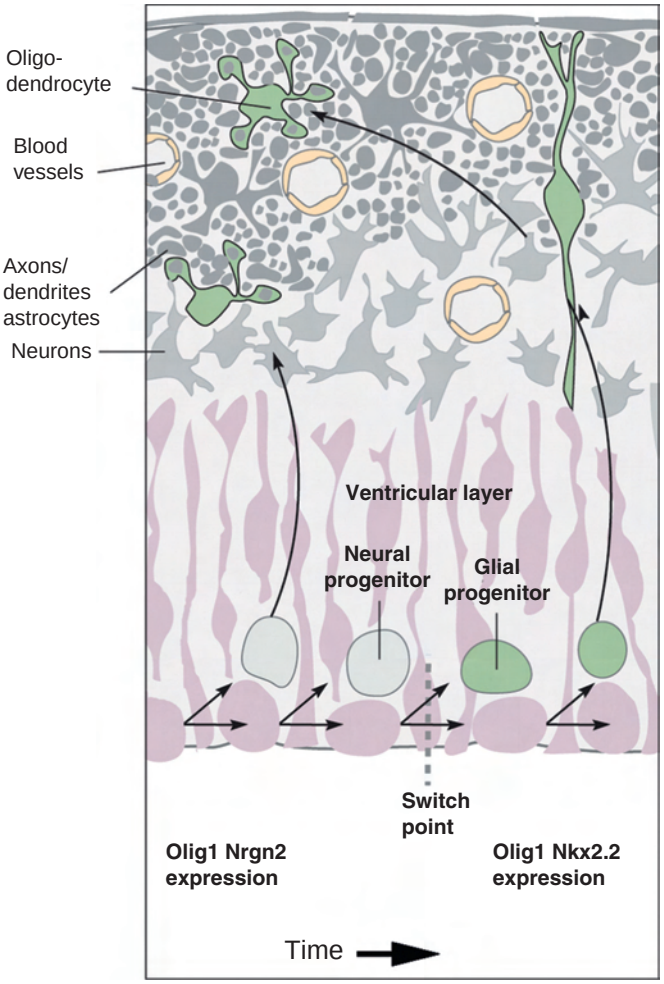

Fig. 3.11 Oligodendrocyte development in vertebrates. Oligodendrocytes and neurons are derived from the same pool of stem cells that divide in the ventricular layer of the developing neural tube (pMN). At early stages, when the progenitors express both Olig1/2 and Neurog2, they generate motor neurons. Later in development, the same population of progenitors begin to express Nkx2.2, and down-regulate their expression of Neurog2. This acts as a molecular switch to cause the cells to start making oligodendrocytes instead of motor neurons.

Fig. 3.12 Drawing of a Golgi stained section showing the neurons in the human cerebral cortex from Ramón y Cajal, 1952. The layering and complex dendritic processes are critical for the processing units of the cerebral cortical columns.

depending on their function. For example, regions devoted to processing sensory information, like the visual cortex, have relatively large numbers of layer IV cells, which form the input layer, while regions important in the "output" of information from the cortex, such as the primary motor cortex, have large layer V pyramidal neurons, and relatively few layer IV cells. As the size of the cortex increased in evolution, there was an increase in specialization of the various regions of cortex, such that in people there are as many as 50 different regions that can be identified on the basis of their distinct "cytoarchitecture" (i.e., differences in the relative numbers of neurons in each of the six layers; see Chapter 2). So what are the mechanisms that have enabled us to develop the amazing neural tissue responsible for the accomplishments of Shakespeare and Einstein?

As noted in the previous chapter, the cerebral hemispheres develop from the wall of the telencephalic vesicle. The neocortex begins as a relatively simple neuroepithelium, similar to that which we have already encountered in the posterior regions of the neural tube—the spinal cord. The early embryonic neocortex is made up of morphologically homogeneous cells that span the width of the epithelium and have a simple bipolar shape and undergo extensive rounds of mitosis as the cerebral vesicle expands (**Figure 3.13**). Within a few days (in the mouse) additional cell types can be identified in the developing cerebral cortex, including postmitotic, migrating neurons and additional types of proliferating cells. The two most well-characterized types of proliferating cells are the apical progenitors (also called the radial glia, for reasons described below) and the intermediate precursor cells (IPCs or basal progenitors). The apical progenitors act as the primary "stem cell" of the cerebral cortex, with the capacity to generate all types of neurons and glia (see below). They divide asymmetrically in the region of the cortex adjacent to the lateral ventricle (a.k.a., the ventricular zone) and one of the cells from the mitotic division is a postmitotic neuron, which migrates along the basal process of the apical progenitor to its final position in one of the layers of the cortex (Figure 3.13).

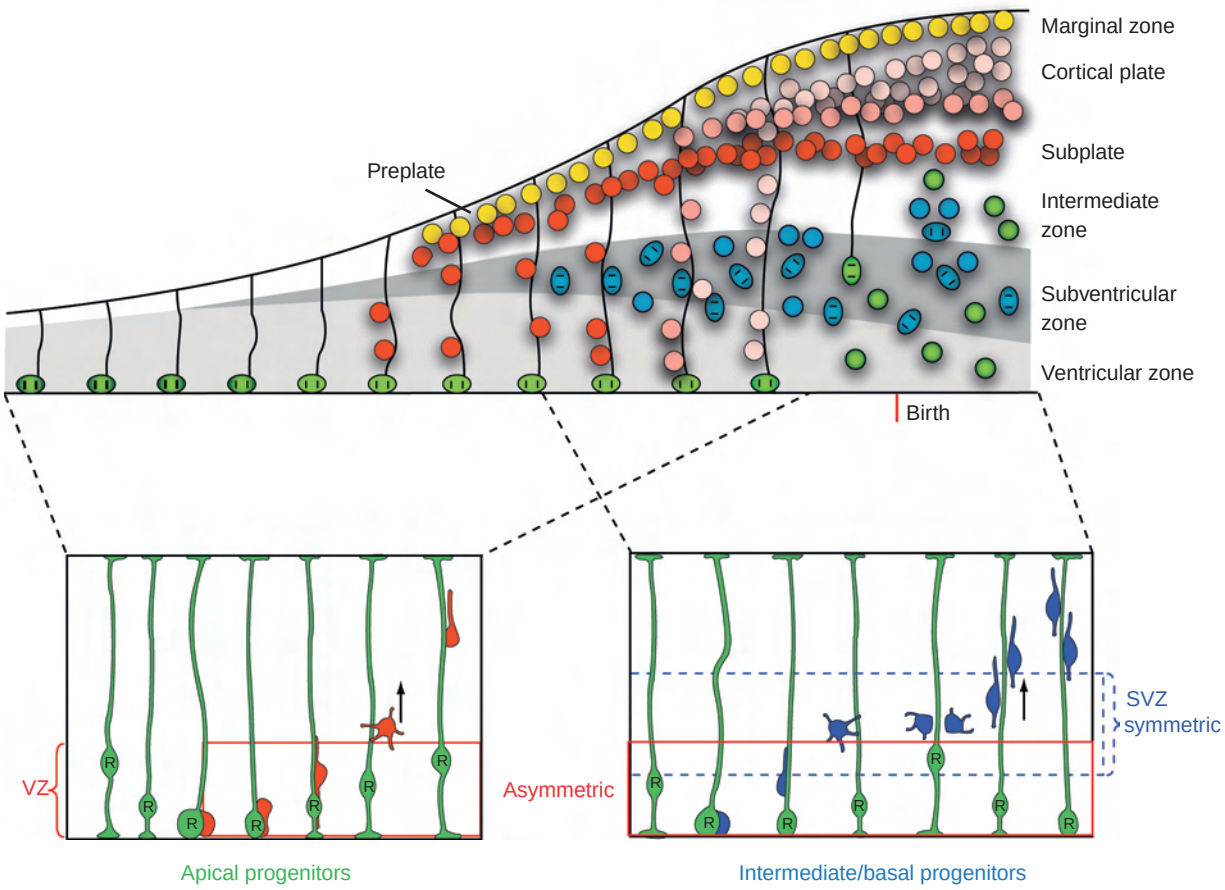

Fig. 3.13 Histogenesis in the cerebral cortex proceeds through three stages. In the first stage of histogenesis, the wall of the cerebral cortex is made up of the progenitor cells, which occupy the ventricular zone (VZ). In the next stage of development, the first neurons exit the cell cycle (red) and accumulate in the preplate, adjacent to the pial surface. The neurons of the preplate can be divided into the more superficial Cajal-Retzius cells and the subplate cells. In the next stage of cortical histogenesis, newly generated neurons (red) migrate along radial glial fibers to form a layer between the Cajal-Retzius cells and the subplate. This layer is called the cortical plate, and the majority of the neurons in the cerebral cortex accumulate in this layer. *(Modified from Noctor et al., 2004; 2008)*

The first neurons that are generated from the ventricular zone migrate a short distance to form a distinct layer known as the preplate, just beneath the outer surface of the cortex (Figure 3.13). The preplate consists of two distinct cell types: a more superficial marginal zone, containing a group of large, stellate-shaped cells, known as Cajal-Retzius cells, and a deeper zone of cells, called the subplate cells. The next stage of cortical development is characterized by a large accumulation of newly postmitotic neurons within the preplate (Marin-Padilla, 1998). These new neurons are called the cortical plate. The cortical plate divides the preplate into the superficial marginal zone (Cajal-Retzius cells) and the intermediate zone (subplate cells and increasing numbers of incoming axons). The developing cortex is thus described as having four layers: the ventricular zone, the intermediate zone, the cortical plate, and the marginal zone (Figure 3.13).

Although many of the mitotic divisions of the apical progenitors in the ventricular zone generate postmitotic cortical neurons directly, others produce progeny that can continue to undergo additional mitotic divisions after they leave the ventricular zone. As noted above, these cells are called intermediate progenitor cells (IPCs) and after leaving the ventricular zone, they migrate a short distance to a specialized zone between the ventricular zone and the neurons of the cortex called the subventricular zone (or SVZ; Figure 3.13). Once an IPC has migrated to the SVZ, it divides symmetrically and usually generates two neurons, but may divide up to three times, making as many as six neurons.

The next phase of cortical histogenesis is characterized by the gradual appearance of defined layers within the cortical plate. As increasing numbers of newly generated neurons migrate from the ventricular zone into the cortical plate, they settle in progressively more peripheral zones. Meanwhile, the earlier-generated neurons are differentiating. Thus, later-generated neurons migrate past those generated earlier. As noted earlier in the chapter, this results in an inside-out development of cortical layers (Figure 3.3).

Pasko Rakic (1988) hypothesized that the progeny of each apical progenitor (ie. radial glial cell) form a column of differentiated neurons. As mammals evolved, they acquired more and more of these radial columns to expand the cortical processing power. This rather simple amplification strategy could explain the relative ease with which this region of the brain can undergo remarkable expansions during evolution. For example, the difference between monkey and human cortex can be viewed as a tenfold increase in the number of these

radial units. This could come about as a result of symmetric mitotic divisions of the apical progenitor cells to expand their number and the ventricular surface they occupy. As the number of these units increases, the resulting increase in cerebral cortical surface area requires that sulci and gyri form and the cortex becomes folded. The radial unit hypothesis has recently been modified to include the IPCs (Pontious et al., 2008). Changes in the IPC amplification factor (1–3 cycles) at different times of development in different regions of cortex might explain the differences in cell numbers in the different areas of cortex, and might also provide a mechanism for the variation in relative cell numbers in the various regions of cerebral cortex. The way this could work for motor cortex, a region with many layer V projection neurons, versus visual cortex, a region with relatively more layer IV input neurons, is as follows: 1. during the early stage of neurogenesis, when layer V cells are being made, the IPCs in the motor cortex divide more times and therefore generate extra layer V neurons; 2. in the sensory cortex, however, the IPCs do not divide as many times early in cortical development, but instead have most of their divisions during the middle stage of cortical development, when layer IV neurons are generated. In the last part of neurogenesis, both regions have the same number of IPC divisions, and so have the same number of upper layer (2–3) neurons. The final cytoarchitecture of the two regions reflects the difference in timing of maximum IPC proliferation, with the sensory area having more layer IV input neurons and the motor cortex having more layer V output neurons. A great deal more will be said about the molecular mechanisms that specify the various types of neurons in the cerebral cortex in Chapter 4.

The neurons produced in the ventricular zone then have to migrate to their final destinations in the different layers of the cerebral cortex. Since the processes of the apical progenitor cells span the entire thickness of the cortex, the cortical neurons that are generated use the predominantly radial orientation of their processes to guide their migration. Serial section electron microscopic studies by Rakic first clearly demonstrated the close association of migrating neurons with the processes of these cells, though at the time they were thought to be glial cells and so were given the name, "radial glia" (**Figure 3.14**). The migrating neurons wrap around their processes like you would if you were climbing up a pole. It has been possible to

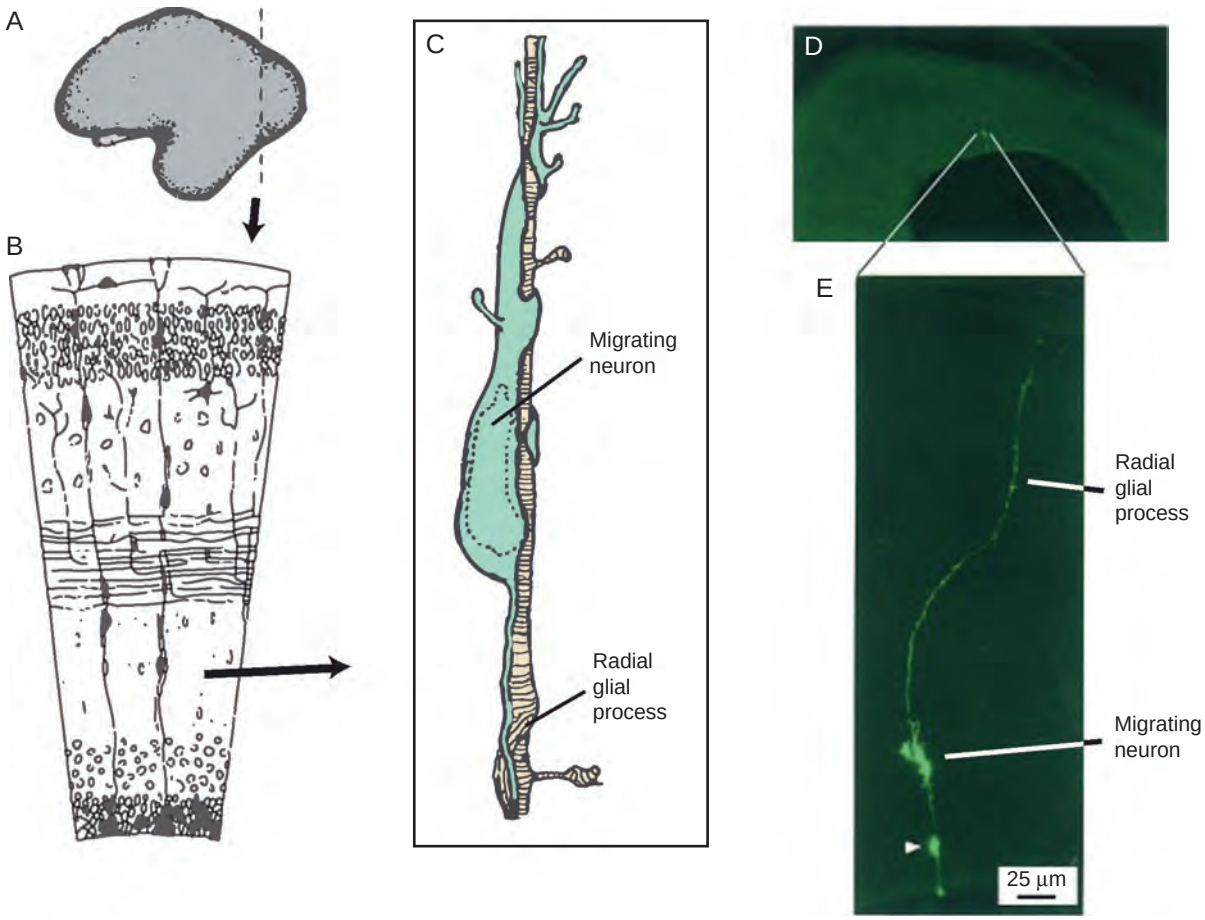

Fig. 3.14 A.B. Migration of neurons along radial glia. The radial glial fibers extend from the ventricular zone to the pial surface of the cerebral cortex. A section through the cerebral cortex at an intermediate stage of histogenesis shows the relationship of the radial glia and the migrating neurons. C. The postmitotic neurons wrap around the radial glia on their migration from the ventricular zone to their settling point in the cortical plate. *(From Rakic, 1972)* D,E. Live imaging of GFP labeled radial glia shows that radial glia are the same cells as the progenitors. Noctor et al. (2002) used a retrovirus to label small numbers of cortical progenitor cells in slice cultures of the cerebral cortex of mice. In this example, they found that the radial glia (arrowhead) has undergone several cell divisions, and the progeny are migrating immature neurons. The neurons migrate along the radial glia that generated them. *(From Noctor et al., 2001)*

directly observe the process of neuronal migration in vitro in slices of cerebral cortex kept alive in culture for several days. In these studies, cells in the ventricular zone were labeled using a GFP-expressing retrovirus to mark a subpopulation of the newly generated neuroblasts. As these cells left the ventricular zone, their leading processes were visible. Time-lapse imaging of neuroblasts shows clearly that many of the neuroblasts migrate just as predicted from the EM reconstructions of Rakic (Noctor et al., 2002).

While confirming the EM studies of Rakic, the direct visualization of neuronal migration gave rise to a surprise. As noted above, for many years it was thought that the radial glia and the progenitor cells were two separate populations. The radial glia were thought to have been generated early in development, and then as postmitotic cells, providing a scaffold to guide the newly generated neurons to the correct laminar position. However, the time-lapse imaging of the GFP-labeled radial glia revealed a surprising result: Noctor et al. (2002) found that the radial glia themselves *were* the neuronal progenitors. Figure 3.14 shows an example of one of the clones they found. When the slice was viewed on the first day, the labeled cell was a single radial glial cell, with a process extending the entire width of the cerebral cortex; however, as they continued to analyze the clone on subsequent days, they found that the radial glia underwent several cell divisions, and the progeny were not additional radial glia, but migrating immature neurons. These neurons migrated along the radial glia that generated them. In addition to having the morphology of neurons, these migrating neurons labeled for neuron-specific markers, while the radial glial cell that generated them expressed proteins typical of radial glia. This finding, and critical findings from the labs of Magdelena Götz (Malatesta et al., 2000), Nat Heintz and Gord Fischell (Anthony et al., 2004) led to the model that was presented earlier in this section, that the radial glia and the ventricular zone apical progenitors are one and the same, and most neurons in the cortex are derived from them.

In addition to the predominantly radial migration of the newly generated neurons, however, there are also some cells that migrate tangential to the cortical surface, in the intermediate zone. Some of these cells arise in the ventricular zone from the cortical progenitors, but most of them are not derived from the cortical ventricular zone at all, but instead migrate all the way from the ventricular zone of a subcortical forebrain region (**Figure 3.15**). These tangentially migrating neurons are a special subpopulation of the neurons in the cortex. Although most of the neurons of the cerebral cortex are pyramidal in shape and use the neurotransmitter glutamate, there are other populations of neurons in the cerebral cortex that are stellate-shaped and use GABA as their transmitter. These GABA+ cells are not derived from the cortical ventricular

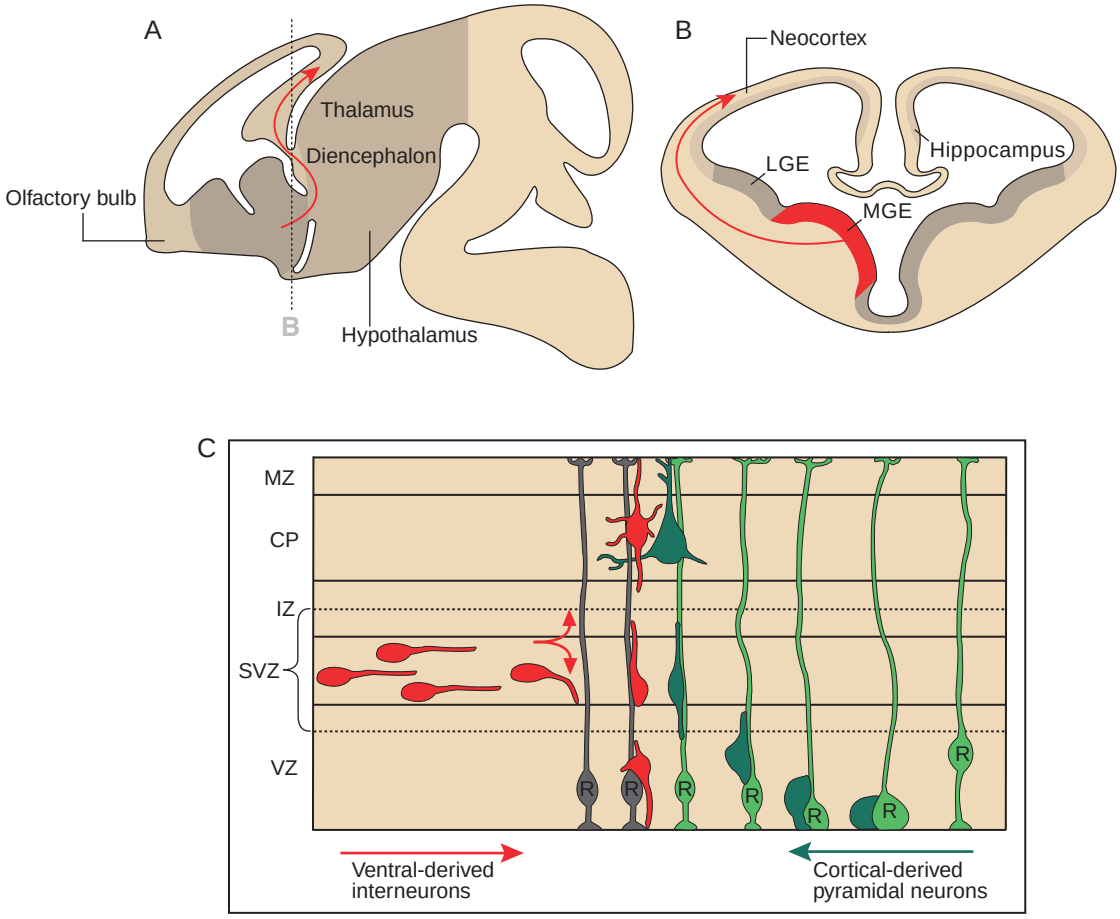

Fig. 3.15 The neurons of the cerebral cortex derive from both intrinsic and extrinsic sources. Most of the neurons in the cortex are derived from the ventricular zone cells immediately below their adult location. A-C. This figure shows the paths taken by the neurons from the ganglionic eminence in red at low (A,B) and high magnification (C) and the neurons generated within the cortex in green. *(Modified from Kriegstein and Noctor, 2004)*

zone, but instead they are the tangentially migrating cells that were produced by the progenitors in the subcortical zone known as the medial ganglionic eminence (MGE). Although the primary role of the MGE during development is to produce the neurons and glia of the basal ganglia (deep forebrain nuclei), they also produce these special neurons for the cortex. This was directly shown by the following experiment: when cortical slices were cultured without the MGE attached, the number of GABA neurons in the cortex was greatly reduced as compared to cultures that contained the MGE. The migration of these cells has also been directly visualized by labeling the premigratory population in the MGE, and tracking their migration to the cerebral cortex. Thus, it is now accepted that the precursors of most GABA-containing interneurons in the cerebral cortex migrate all the way from the subcortical progenitor zones (Corbin et al., 2001; Nakajima, 2007).

CEREBELLAR CORTEX HISTOGENESIS

As noted above, the cerebellum is a large, highly convoluted part of the brain that is critical for control of our movements, particularly our balance. Cerebellar function is particularly susceptible to ethanol; the weaving motion of alcoholics is likely due to alcohol's effects on cerebellar function. The mature cerebellum is made up of several distinct cell types, each repeated in an almost crystalline array (**Figure 3.16**). The two most distinctive of these cell types are the giant Purkinje cells and the very small granule cells. Purkinje cells are the principal neurons of the cerebellar cortex, sending axons out of the cortex to the deep cerebellar nuclei. The cerebellar granule neurons are much more numerous than the Purkinje cells. In fact, the cerebellar granule cells are the most numerous type of neuron in the brain. In the mature cerebellum, they form a layer deep to the Purkinje cells, and their axons extend past the Purkinje cell layer into the molecular layer. The axons of the granule cells bifurcate in the molecular layer, into a T-shape, and these axons extend in the molecular layer for a considerable distance, synapsing on the Purkinje cell dendrites. One can think of the Purkinje cells as telephone poles and the granule cell axons as the telephone wires.

The generation of the intricate cerebellar architecture is a complex process. The large Purkinje neurons are generated from a ventricular zone near the fourth ventricle of the brainstem, in a manner similar to the way in which the neurons of the cerebral cortex are produced. Once they have finished their final mitotic division, the Purkinje cells migrate a short distance radially to accumulate as an irregular layer, known as the cerebellar plate. As the cerebellum expands, these cells become aligned to form a single, regularly spaced layer. The Purkinje cells then grow their elaborate dendrites. In addition to the Purkinje cells, the ventricular zone generates several other cerebellar interneurons, such as the stellate and basket cells. In contrast to the somewhat standard pattern of neurogenesis of the Purkinje cells and the stellate and basket cells, the granule cells arise from a completely separate progenitor zone, known as the rhombic lip (**Figure 3.17**). The granule cell precursors are initially generated near the rim of the fourth ventricle but then migrate away from the ventricular zone, over the top of the developing Purkinje cells to form a secondary zone of neurogenesis, called the *external granule layer*. The cells in this layer continue to actively proliferate, generating an enormous number of granule cell progeny, thus increasing the thickness of the external granule layer considerably. The external granular layer persists for a considerable

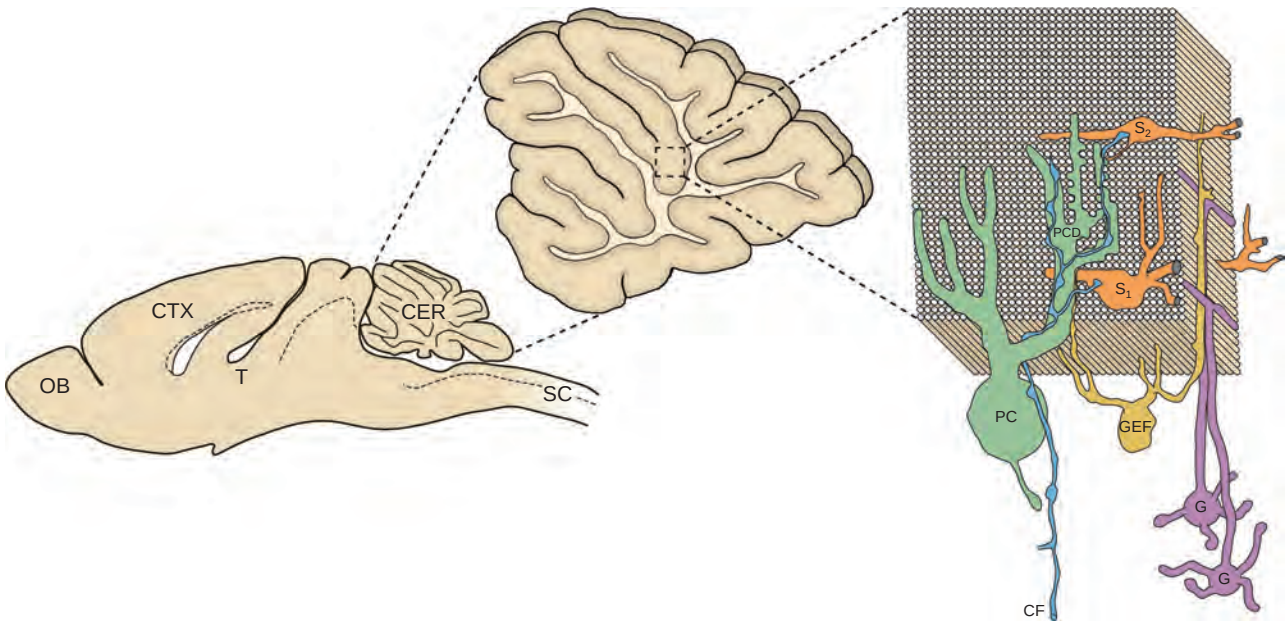

Fig. 3.16 The neurons of the cerebellar cortex are arranged in a highly ordered fashion. In the mature cerebellum, the very large Purkinje cells (PC) lie in a single layer (P) and have an extensive dendritic elaboration that lies in a single plane. The granule cells (red) lie below the Purkinje cells in the granule cell layer (purple) (G) and have a T-shaped axon that runs orthogonal to the plane of the Purkinje cell dendrites, like phone wires strung on the Purkinje cell dendritic "poles" in the molecular layer (M). In addition to these distinctive cell types, the cerebellar cortex also contains other cell classes, the stellate cells (S) and the Golgi epithelial cells (GECs). *(Modified from Rakic, 1971b)*

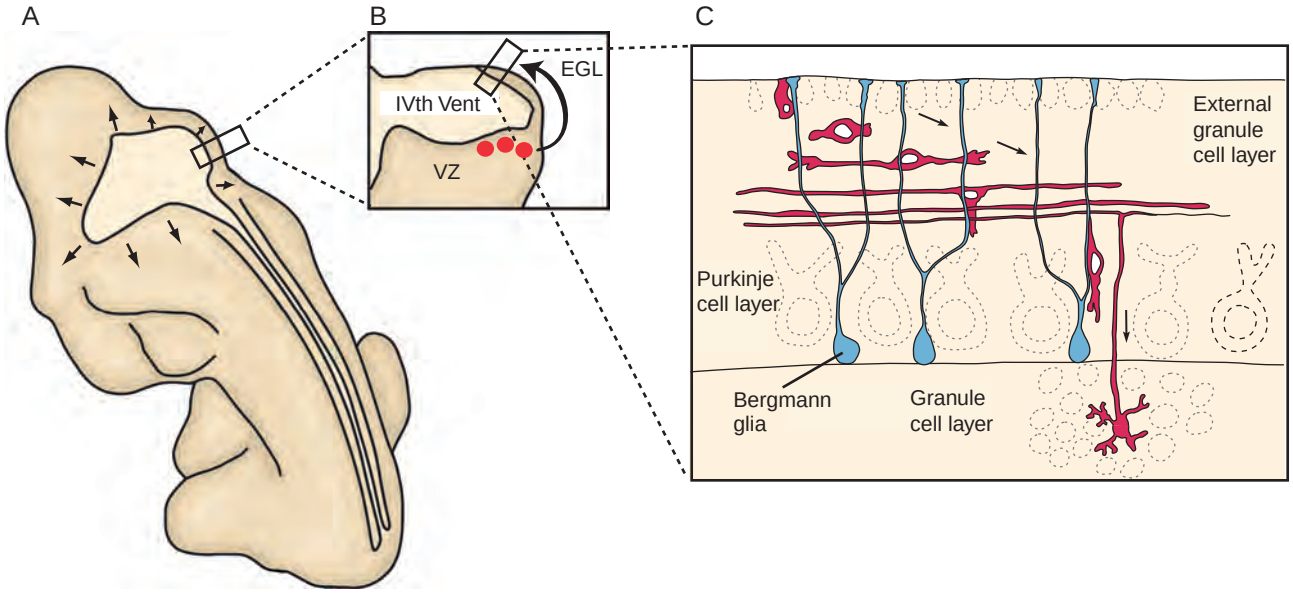

Fig. 3.17 The precursors of the cerebellar granule cells come from a region of the rhombencephalon known as the rhombic lip. The rhombic lip is a region of the hindbrain that lies adjacent to the fourth ventricle. Cells from this region migrate over the surface of the cerebellum to accumulate in a multicellular layer—the external granule cell layer. A.B. This dorsal view of the developing brain shows the migratory path of the granule cell precursors from the rhombic lip of the rhombencephalon to the surface of the cerebellum (arrows). Granule cell production in the external granule cell layer is followed by the migration of these cells to ultimately lie deep to the Purkinje cell layer. C. Arrows show the migratory path a single neuron would take from its birth to the granule cell layer. The Bergmann glial cells are shown in blue and function as guides for the migrating neurons. The migration of a granule cell is thought to take place along a single glia fiber, but in the diagram the migrating neuron is shown to be associated with several glial cells for clarity. *(Modified from Ramón y Cajal, 1952)*

time after birth in most mammals and continues to generate new granule neurons. There are still granule neurons migrating from the external granule layer as late as two years after birth in humans (Jacobson, 1978).

Although the granule neurons are generated superficially in the cerebellar cortex, they come to lie deep to the Purkinje cells in the mature cerebellum. The developing granule neurons must therefore migrate past the Purkinje cells. Figure 3.17 shows what this process looks like, as originally described by Ramón y Cajal (1952). Soon after their generation, after their final mitotic division, the granule cells change from a very round cell to take on a more horizontal-oriented shape as they begin to extend axons tangential to the cortical surface. Next, the cell body extends a large process at right angles to the axon. As this descending process grows deep into the cerebellum, the cell body and nucleus follow, leaving a thin connection to the axon. Meanwhile, the axons have been extending tangentially, and so the cell assumes a T-shape. The cell body eventually migrates past the Purkinje cell layer and then begins to sprout dendrites in the granule cell layer. The migration of the granule cells is another example of the importance of radial glia in CNS histogenesis. As they migrate, a specialized type of radial glia, known as the Bergmann glia, guides the granule cells. EM studies, similar to those described for the cerebral cortex, first demonstrated the relationship between the migrating granule cells and the Bergmann glia (Rakic, 1971b). Throughout the migration of the granule cells, they are closely apposed to the Bergmann glial processes. Hatten and her colleagues (1985 and 1990) have been able to demonstrate directly the migration of granule cells on Bergmann glia using a dissociated culture system. When the external granule cell layer is removed from the cerebellum and the cells are cultured along with cerebellar glia, the granule glial cells migrate along the extended glial fibers in vitro. Time-lapse video recordings have even captured the granule cell migration in action.

A factor first encountered in the context of patterning the nervous system (Chapter 2), Sonic hedgehog, is also a key mitogen for nervous system progenitors, and this has been best demonstrated in the cerebellum. The way in which Shh acts in neurogenesis demonstrates the way in which differentiated neurons can feed back on the progenitors to maintain their proliferation and ensure that the correct number of neurons is generated during development (Wechsler-Reya and Scott, 1999). The Purkinje cells produce the mitogen, Shh, while the granule cell progenitors express the Shh receptors, patched and smoothened (named for *Drosophila* mutants defective in the homologous genes). The Shh released from the Purkinje cells stimulates the granule cell progenitors to make more granule cells. If the Shh pathway is experimentally blocked, fewer granule cells are produced. If the Shh pathway is activated, granule cell production is increased. In this way, Shh is used by the developing nervous system to mediate the cell interactions between the differentiated Purkinje neurons and the neural progenitors. This pathway also provides another example of how a childhood tumor can result from a misregulation of neurogenesis. Children with mutations in the Shh receptor, *patched*, that mediates Shh signaling will develop a tumor called medulloblastoma, in which granule cell production is fatally uncontrolled (Goodrich et al., 1997).

MOLECULAR MECHANISMS OF NEURONAL MIGRATION

As the previous two sections have shown, the processes of neurogenesis and cell migration are frequently closely coupled in the developing brain. In both the cerebral cortex and the cerebellum, the process of glial-guided migration has been the subject of very intense investigation over more than 20 years. In this section, we will describe some of what is known about the molecular mechanisms that underlie the correct positioning of neurons in laminated structures like the cerebral and cerebellar cortices.

Some of the greatest advances in our understanding of the molecular mechanisms of cell migration have come about by analysis of naturally occurring mouse mutations that disrupt the normal migration of neurons. One important function of the cerebellum is to maintain an animal's balance. Lesions to the cerebellum in humans frequently produce a syndrome that includes unsteady walking, known as ataxia. Genetic disruptions of the cerebellum in mice produce a similar syndrome, and therefore they can be identified and studied. By screening large numbers of mice for motor abnormalities, several naturally occurring mutations have been identified that disrupt cerebellar development (Caviness and Rakic, 1978). Because of the nature of the symptoms, these mutant mouse strains have names like *reeler*, *weaver*, and *staggerer*. The mutant genes that underlie these phenotypes have been identified, and one of these mutants, *reeler*, has been particularly informative in understanding neuronal migration. The *reeler* mutant mouse has ataxia and a tremor. Histological examination of individually labeled neurons in *reeler* mutant cerebral and cerebellar cortex revealed gross malpositioning of the cells. In the cerebellar cortex, the Purkinje cells are reduced in number and, instead of forming a single layer, appear to form aggregates instead. There are fewer granule cells, and most of them fail to migrate from the external granule cell layer to their normal mature position below the Purkinje cells.

The effects of the *reeler* mutation have been particularly well-studied in the cerebral cortex, where instead of the normal inside-out pattern of neurons that was described in the previous section, in the *reeler* mutant the later generated neurons fail to migrate past those generated earlier and so the mice have an outside-in organization of their cerebral cortex (**Figure 3.18**). The defective molecule underlying the *reeler* phenotype was identified several years ago. It is a large glycoprotein, named Reelin, containing over 3000 amino acids, and it bears similarities to some extracellular matrix proteins (D'Arcangelo et al., 1995). The Reelin protein is expressed by the most superficial neurons of the cortex, the Cajal-Retzius cells. Insight into the molecular mechanisms by which Reelin controls migration have come from the identification of additional components in its unique signal transduction pathway. Mutations in the genes coding for a tyrosine kinase called disabled or Dab1, the VLDLR (very low density lipoprotein receptor), and ApoER2 (apolipoprotein E, receptor 2) all cause defects in cerebral cortical neuroblast migration similar to those found in *reeler* mice (Jossin et al., 2003), where the newly generated neurons fail to move past the previously generated ones. The VLDLR, along with ApoER2, form a receptor complex that phosphorylates the Dab1 protein upon Reelin binding. Once phosphorylated, disabled can recruit other second messengers in the tyrosine kinase pathway and activate a host of cellular

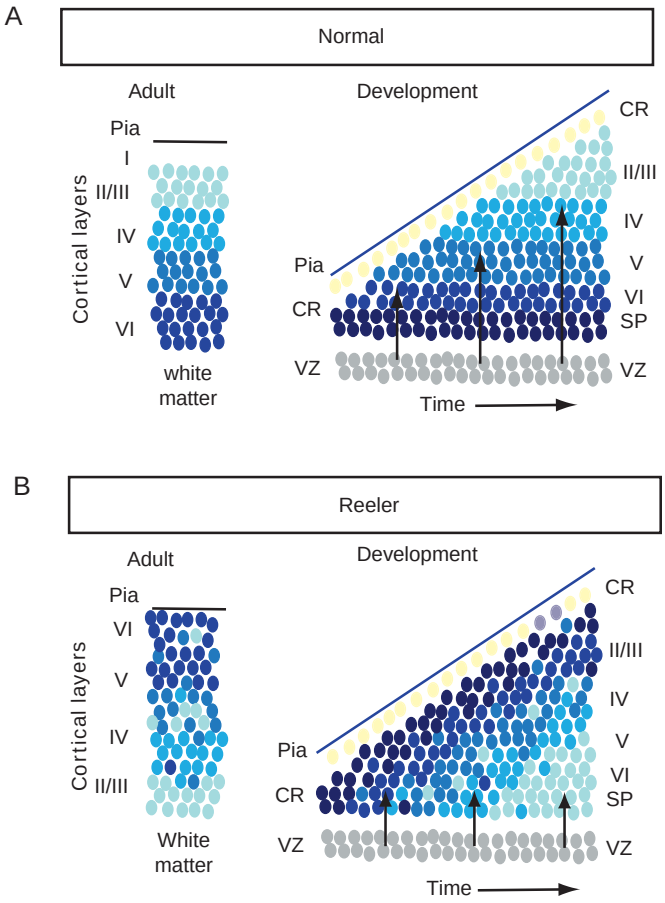

Fig. 3.18 The function of Reelin in the cerebral cortex. A. In normal cortical development the early generated neurons bypass the previously generated neurons to produce the inside-out developmental ordering to the lamination. B. In *reeler* mice, this orderly lamination process is disrupted, and the newly generated neurons cannot pass the previous ones and the lamination is reversed. *(Modified from Cooper, 2008)*

responses. The VLDLR and ApoER2 receptors are expressed in the migrating neuroblasts and in the radial glia themselves, while Reelin is made by the Cajal-Retzius cells at the cortical surface. The observed cellular expression pattern of Reelin and its receptors has led to two basic classes of hypotheses for its function during cortical development: (1) Reelin might be a chemoattractant in the cerebral cortex, causing the migrating neuroblasts to move toward the source of Reelin in the Cajal Retzius cells in the superficial layers of the cortex; (2) alternatively, Reelin could act as a stop signal at the cortical surface, telling the neuroblasts to "get off the track" and form a new cortical layer. Many experiments have been carried out to test these ideas, both in vivo and in vitro, but until recently, no clear answer emerged, and there was support for and against both hypotheses. For example, in support of the second hypothesis, Dulabon et al. (2000) found that adding Reelin to migrating neuroblasts in cell culture causes them to stop their migration. However, this result is not inconsistent with Reelin playing a role as a chemoattractant, since adding Reelin to cell culture surrounds the migrating neuroblasts with the potential attractant and thus causes them to be attracted equally in all directions and to stop moving. To distinguish

between these two possibilities, Curran's group generated a transgenic mouse that has Reelin expressed under the control of the Nestin promoter, and therefore expressed in the radial glia themselves (Magdeleno et al., 2002). These mice express Reelin all along the path of migration of the neurons, including the Cajal-Retzius cells. If Reelin is a stop signal or an attractant, the migrating neuroblasts should never leave the ventricular zone. However, they found that the Nestin-Reelin mice were essentially normal. The migrating neuroblasts still left the ventricular zone on schedule and in fact made basically normal layers. They also mated the Nestin-Reelin mice with *reeler* mice. These mice only have the Reelin in the radial glia and no longer express any Reelin in the Cajal-Retzius cells. Although the cortical lamination was not perfect, it was improved over that of the *reeler* mouse. Therefore, it looks as if it is less important where the Reelin is localized in the developing cortex, as long as there is some Reelin around.

More recently, studies on the components of the Reelin signal transduction pathway have shed some light on this puzzle. Most signaling pathways have a built-in negative feedback mechanism to limit the time that the signal is active in a cell. This is particularly important in development, where timing can be critical. For the Reelin pathway, once the Dab1 has been activated, it is targeted to the proteosome by a protein called Cullin5 (Cul5), and then degraded. The Cooper lab has knocked down the level of Cul5 in the migrating neurons (Feng et al., 2007), which caused the activated Dab1 to persist in the neurons longer than it normally would. In these mice, the migrating neurons fail to stop once they have passed the previously generated one, and overshoot their target layer to end up with the Cajal-Retzius cells. Since loss of Dab1 entirely leads to a failure of the newly generated neurons to migrate past the previous layer, while over-activating the system causes them to migrate too far, the simplest interpretation is that Reelin initially causes the activation of Dab1 to promote neuronal migration, but then causes Dab1 degradation, which causes the cells to stop. In this way, Reelin can be both a "go" signal and a "stop" signal (**Figure 3.19**).

In addition to Reelin, a large number of molecules have been implicated in neuroblast migration in the cerebral and cerebellar cortices, including astrotactin, integrins, and neuregulins. Integrins are cell adhesion molecules that allow many different types of cells to attach to the proteins in the extracellular matrix. Since these adhesion receptors are necessary for the pial extracellular matrix formation, it is not surprising that they are required for the appropriate formation of the glial scaffold and hence the migration of the neuroblasts and correct positioning of the cerebral cortical neurons. It is as if you were trying to climb a ladder without a wall to lean it against. Another class of molecules, the neuregulins and their receptors, likely has a very different role. Neuregulin, or glial growth factor, activates receptor tyrosine kinases called ErbBs on the glial cell surfaces and promotes the appropriate differentiation and/or survival of the glial cells. Without the glial cells adopting their elongate morphology, the neuroblast migration is abnormal. Again comparing this with a ladder, it is as if you were trying to climb a ladder made of rubber.

In sum, many cellular and molecular interactions are necessary for proper arrangement of the neurons in the complex neuronal structures that make up the mature brain. Nearly all the neurons in the brain end up some distance from where they were generated in the ventricular zone, and the mature neuronal

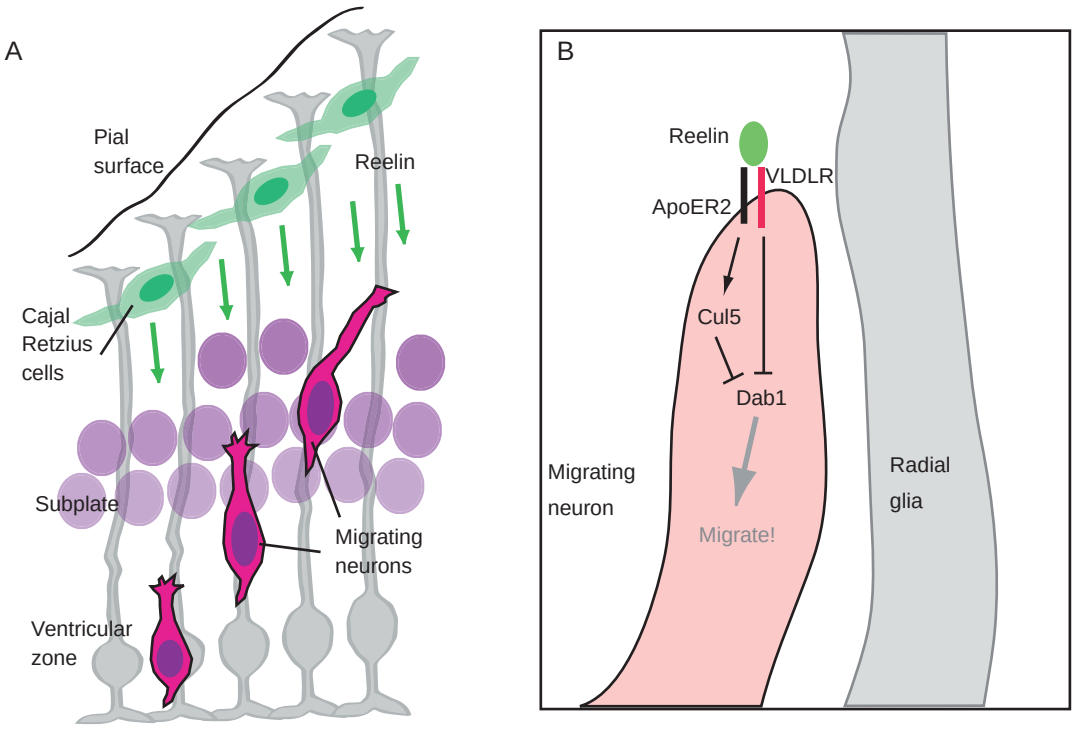

Fig. 3.19 A. Reelin, the defective molecule underlying the *reeler* phenotype, is expressed by the most superficial neurons of the cortex, the Cajal-Retzius cells (green). B. Higher magnification view of the leading process of a migrating neuron to show the signaling complex. Dab1 promotes cell migration. Binding of Reelin to ApoER2 and VLDLR results in the phosphorylation and ubiquitinlyation of Dab1, which leads to its degradation and inhibition of migration.

circuitry depends on cells getting to the right place at the right time. Mice with mutations in genes critical for neuronal migration have motor deficits, but it is likely that more subtle deficits are caused by less dramatic changes in neuronal migration. Several inherited mental retardation syndromes in humans are now known to be caused by defective migration of cortical neuroblasts. The beautiful choreography of neuronal migration is clearly an essential part of building a working nervous system (see Box 3.1).

POSTEMBRYONIC AND ADULT NEUROGENESIS

The process of neurogenesis ceases in most regions of the nervous system in most animals. Neurons themselves are terminally differentiated cells, and there are no well-documented examples of functional neurons reentering the mitotic cycle. However, it has long been appreciated that in most species some new neurons are generated throughout life. There is considerable remodeling of the nervous system of insects during metamorphosis. Much of this remodeling occurs through cell death, but new neurons are also produced.

Many amphibians also go through a larval stage. Frogs and toads have tadpole stages where a considerable amount of body growth takes place prior to metamorphosis into the adult form. During larval stages, many regions of the frog nervous system continue to undergo neurogenesis similar to that in embryonic stages. One of the most well-studied examples of larval frog neurogenesis is in the retinotectal system. The eye of the tadpole, like that of the fish, increases dramatically in size after embryonic development is complete. During this period, the animal uses its visual system to catch prey and avoid predators. The growth of the retina, however, does not occur throughout its full extent, but rather is confined to the periphery (**Figure 3.20**). Similar to the way in which a tree grows, the retina adds new rings of cells at the preexisting edge of the retina. This provides a way for new cell addition to go on at the same time the central retina functions normally. As the new retinal cells are added, they are integrated into the circuitry of the previously differentiated retina, into a seamless structure. The zone of cells responsible for adding the new neurons at the edge of the frog retina is called the ciliary marginal zone or CMZ. The cells of this region act as true "retinal stem cells," in the sense that they can generate all the different types of retinal neurons, and they seem to be inexhaustible: most of the retina of the mature frog or fish is actually generated by these cells, not by the embryonic progenitor cells of the retina. The CMZ is organized in a gradient with the most primitive stem cells located most peripherally, next come the multipotent progenitors, and then most centrally are the differentiating neurons, immediately adjacent to the mature retina. At the same time that new cells are added to the peripheral retina, the optic tectum also adds new neurons. The coordination between neurogenesis in these two regions likely involves their interaction via the retinal ganglion cell projection of the tectum. The growth of the optic tectum, the brain center to which the retina sends its axons, occurs at its caudal margin, so the axons of the ganglion cell must shift caudally during this time.

One of the most well-studied examples of neurogenesis in mature animals comes from studies of song birds. In 1980, Fernando Nottebohm reported that there was a seasonal change in the size of one of the brain nuclei important for song production in adult male canaries. In songbirds, specific nuclei in the telencephalon of the brain are critical for the production of the song. The HVC nucleus is of particular importance for both song learning and song production (see Chapter 10). The HVC is almost twice as large in the spring, when male canaries are generating normal adult song, than in the Fall, when they no longer sing. Nottebohm initially proposed that this change in size might be due to seasonal changes in the numbers of synapses. In further studies of the HVC in male and female canaries, Nottebohm also noticed that it was larger in males, which learn complex songs, than in female birds, which do not sing. Moreover, if adult females were given testosterone injections, the HVC nucleus grew by 90% and the female birds acquired male song (Nottebohm, 1985; 2002); later studies demonstrated that ^{3}H-thymidine neurons were in fact generated in the mature bird (Paton and Nottebohm, 1984).

To determine whether new neurons were added to the nucleus in response to the testosterone, female birds were injected with ^{3}H-thymidine as well as testosterone, and the animals were sacrificed for analysis five months later.

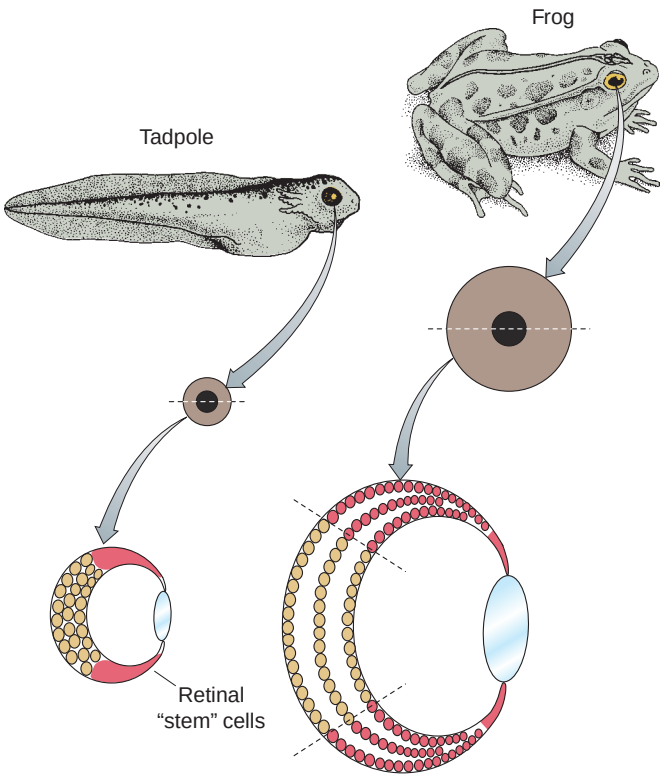

Fig. 3.20 The eyes of frogs grow by the addition of new cells to the margin. The neural retina of the frog tadpole is derived from the neural tube, as described in a previous chapter. The initial retinal neurons are generated during embryogenesis. However, as the eye grows, the neural retina grows by means of a specialized ring of retinal stem cells at the peripheral margin of the eye (red). The retinal stem cells generate all the different types of retinal neurons to produce new retina that is indistinguishable from the retina generated in the embryo, and thoroughly integrated with it. In the newly post-metamorphic *Rana pipiens* frog, nearly 90% of the retina has been generated during the larval stages; all this time the retina has been fully functional. This process continues even after metamorphosis but much more slowly.

The researchers found that in both the testosterone-treated and control birds there were many thymidine-labeled cells, and many of these had morphological characteristics of neurons. They also analyzed birds immediately after the injections and found that the new neurons were not produced in the HVC itself, but rather were generated in the ventricular zone of the telencephalon and migrated to the nucleus, analogous to the way in which the nucleus is initially generated during embryogenesis. Subsequent studies have shown that the newly produced neurons migrate along radially arranged glial processes from the ventricular zone to the HVC (Garcia-Verdugo et al., 1998). Thus, ventricular zone neurogenesis is a normally occurring phenomenon in adult canaries. The progeny of the cells produced in the SVZ migrate to the HVC soon after their generation. There they differentiate into neurons, about half of which differentiate into local interneurons and half into projection neurons, which send axons out of the nucleus to connect with other neurons in the brain and form part of the functional circuit for song learning.

Thus, there appears to be a seasonally regulated turnover of neurons in the HVC and in other song control nuclei in the brain of the adult songbird. The turnover of neurons may correlate with periods of plasticity in song learning. Canaries modify their songs each year; each spring breeding season, they incorporate new syllables into the basic pattern, and then in the late summer and fall they sing much less frequently. Combining ^{3}H-thymidine injections with measures of cell death and overall neuronal number in the HVC over a year, one can see two distinct periods of cell death, and each one is followed by a burst in the number of new neurons in the nucleus. Both of these periods of high neuronal turnover correlate with peaks in the production of new syllables added to the song. The neurogenesis is balanced by cell death, and during periods of new song learning the nucleus adds cells, while during periods when no song is generated, the song-related nuclei undergo regression. Is the rate of neurogenesis in the ventricular zone controlled by the seasonal changes in testosterone in the male birds? When the number of labeled cells in the ventricular zone is compared in testosterone-treated and untreated female birds, there are no differences—indicating that the rate of neurogenesis does not change in response to the hormone. However, it appears instead that the survival of the neurons in the HVC is seasonally regulated—neurons generated in the spring have a much shorter average lifespan than those generated in the fall. Thus, the seasonal changes in neuron number in the songbird HVC are not dependent on changes in the number of newly added cells, but rather relate to seasonally and hormonally regulated differences in the survival of the newly produced neurons.

Neurogenesis also occurs in the mature mammalian brain. Although for many years this view was regarded as somewhat heretical, it has become well-accepted in recent years. The ^{3}H-thymidine birthdating studies of Altman, described at the beginning of this chapter, thoroughly documented the time and place of origin of neurons and glia of many regions of the rodent brain. It was found that many brain neurons are generated after birth in rodents. He next extended the labeling period to the second and third postnatal weeks and found that in one particular region, the olfactory bulb, thymidine-labeled cells were still found up to four weeks postnatally. These cells were generated in the subventricular zone (SVZ) in the forebrain and then migrated to the olfactory bulb (Altman, 1962; Altman and Das, 1965; **Figure 3.21**). These early studies of Altman were discounted, in part, because they could not prove that the cells that were generated in the adult formed functional neurons. More recent studies (Lois and Alvarez-Buylla, 1993; Luskin, 1993; Reynolds and Weiss, 1992) (see below) have now used better methods to confirm Altman's findings that neurogenesis occurs in specialized regions of the adult mammalian brain.

Most of the cells generated in the SVZ during the neonatal period and in mature rodents migrate to the olfactory bulb, in what is known as the rostral migratory stream (RMS) (Figure 3.21; Lois et al., 1996). The new neurons that migrate to the olfactory bulb in the RMS are generated at the lateral ventricles of the telencephalon, by cells with astrocytic properties, sometimes called B cells. These B cells have a single cilium that extends into the ventricle, and another process that contacts nearby blood vessels. Both the blood vessel contact and the cilium in the ventricle are thought to be important for the property of these cells that allow them to persist as "neural stem cells" throughout the animal's lifetime, like those of the frog CMZ (above). The B cells slowly self-renew, but at the same time generate C cells, a transit amplifying population, and these then make the neuroblasts, which migrate through the RMS. When the neuroblasts reach the olfactory bulb, they differentiate as granule cells and periglomerular cells, two types of GABAergic neurons. Although for many years it was thought that only inhibitory interneurons were generated in the adult mouse, recent studies have shown that a small number of excitatory glutamergic neurons are produced in this system as well.

How do these cells manage to migrate all the way from the lateral wall of the telencephalic ventricle to the olfactory bulb? The rostral migratory stream has a fascinating structure. The neuroblasts migrate in chains, along extended astrocyte networks. These networks are complex but in general have rostral–caudal orientation. One might imagine that the association of migrating SVZ cells is analogous to the migration of cortical neurons along radial glia; however, the SVZ cells do not appear to require the glia. The migration of SVZ cells has been termed *chain migration* and is distinct from the migration of neurons along radial glia. The SVZ cells form a chain *in vitro*, even in cultures devoid of glial cells, and migrate by sliding along one another.

In addition to the SVZ, one other region of the adult mammalian brain, the dentate gyrus of the hippocampus, also generates new neurons throughout life. Fred Gage and his colleagues have shown that the new neurons generated in the hippocampus are functionally integrated into the circuitry (Van Praag et al., 2002). To assay the function of the newly generated neurons, they used retroviral labeling in adult rats, similar to that which was described in the beginning of the chapter for labeling progenitors in the developing brain. Since a retrovirus will only infect and integrate into mitotically active cells, they were able to label the mitotically active hippocampal precursors with a retrovirus expressing the green fluorescent protein. When the authors examined the GFP-labeled cells after only 48 hours, the cells had a very immature morphology and resembled progenitors, like those found in the developing brain. However, when the animals were allowed to survive for four weeks, many of the GFP-labeled cells now expressed markers of differentiated neurons. Over the next three months these neurons continue to mature. To what

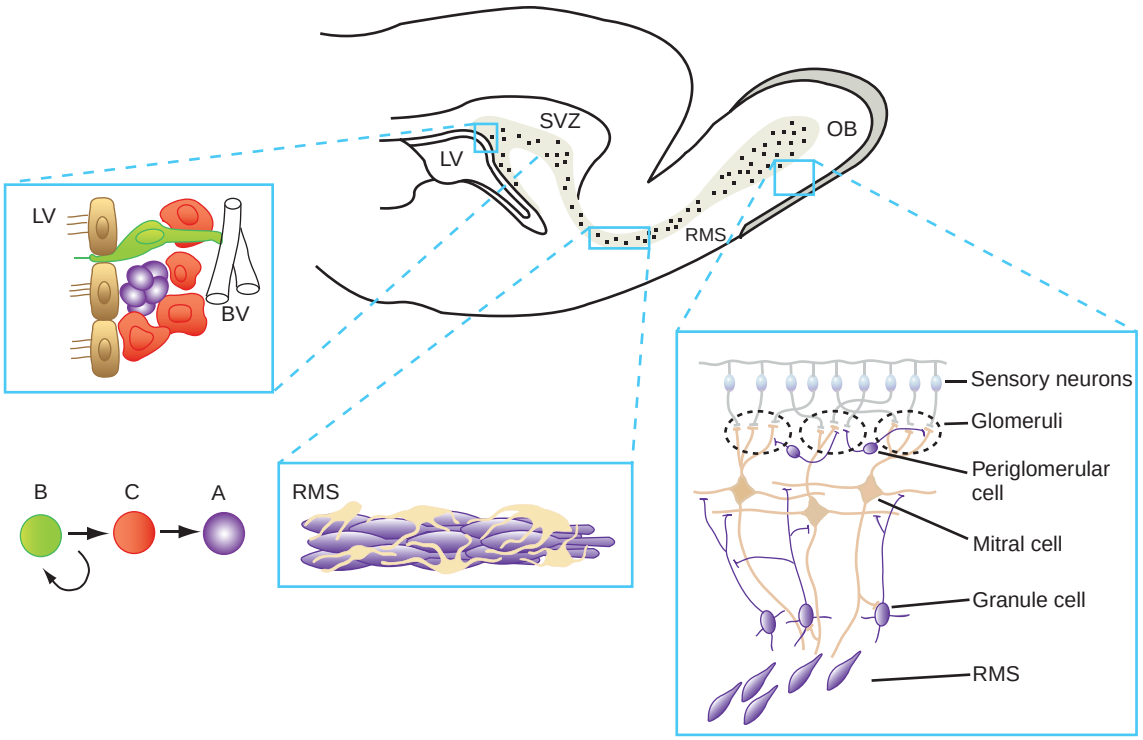

Fig. 3.21 Current model of adult neurogenesis in mice. New neurons are generated from stem cells lining the lateral ventricle (LV) in the subventricular zone (SVZ). The stem cells, which have a single cilium extending into the ventricle, and express GFAP (green) also have a process that contacts a blood vessel (BV). The stem cells, also called B cells, give rise to transit amplifying C cells (red) which then produce the A cells (purple) that migrate to the olfactory bulb via the rostral migratory stream (RMS). When these cells reach the olfactory bulb (OB), they differentiate into either periglomerular cells or granule cells, two types of interneurons in the bulb. *(Modified from Saghatelyan)*

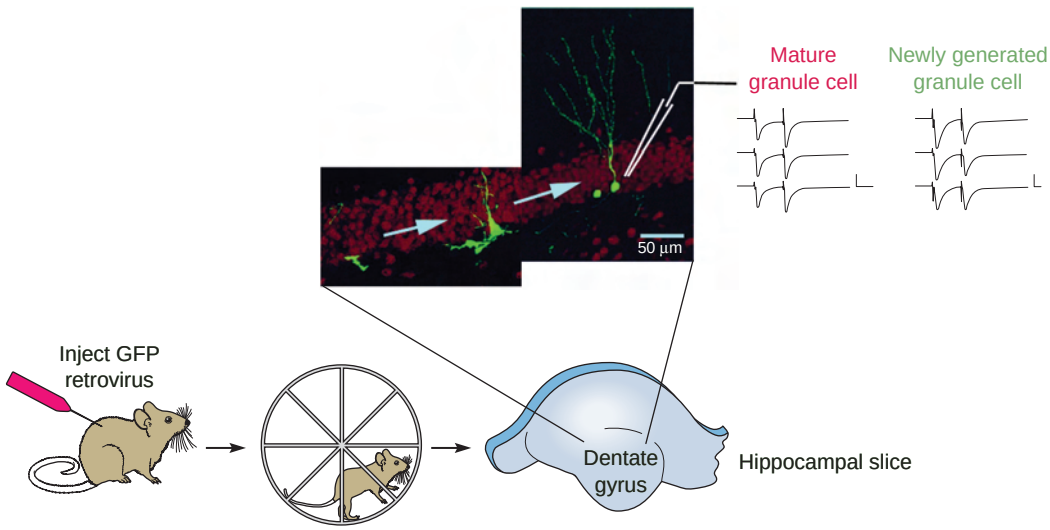

Fig. 3.22 Adult-generated hippocampal neurons are functionally integrated with preexisting neurons. Van Praag et al. labeled proliferating cells in the hippocampus with a GFP-expressing retrovirus, let the animals run on wheels to increase their production of new neurons, and then recorded from the GFP-labeled cells in hippocampal slices. The adult-generated neurons integrated into the hippocampal circuit and showed electrophysiological responses similar to their mature granule cell neighbors. *(Modified from Reh, 2002)*

extent are the new GFP cells functionally integrated into the hippocampal circuitry? The hippocampus can be sliced into thin sections while still functionally active, and the electrophysiological activity of the neurons monitored with microelectrodes (**Figure 3.22**). The newly generated granule cells had electrophysiological properties similar to those found in mature granule neurons, and they receive inputs from the major afferent pathway. Thus, newly generated neurons in the adult hippocampus integrate into the existing circuitry and function like those neurons generated during embryogenesis.

How many of the neurons in the adult brain are generated after we are born? As we have seen above, the most active regions of neurogenesis in the mammalian brain are the hippocampus and the subventricular zone, which produces neurons for the olfactory bulb. To determine what percentage of the neurons in these structures are generated in adulthood, Götz and her colleagues (Ninkovic et al., 2007) used a drug (tamoxifen) inducible form of Cre-recombinase that was specifically targeted to the progenitor cells (and astrocytes). These animals were mated with other mice that had been engineered with a reporter gene (beta-galactosidase) that could be activated by the Cre-recombinase. When the drug tamoxifen was given to the animals at three months of age, the Cre-recombinase was directed to the nucleus where it could induce a recombination of the DNA leading to the expression of the beta-galactosidase, permanently marking all the progeny of the adult progenitor cells. They found that up to one third of the neurons in the glomerular layer of the olfactory bulb were made by the adult stem cells, whereas only 14% of the granule neurons of the hippocampus were generated in adult mice. Since new neurons continue to be produced throughout life by these adult stem/progenitor cells, there must be substantial death of many of the cells in order to maintain a stable ratio of new:old neurons in these brain regions.

What about the rest of our brains? Are new neurons generated during our lives outside of the hippocampus and olfactory bulb? A fascinating "natural" experiment has given insight into this question. During the Cold War, the U.S.A. and the Soviet Union routinely tested nuclear weapons above ground. This led to a global increase in the levels of ^{14}C in the atmosphere until 1963. Frisen's group in Sweden measured the level of ^{14}C in the DNA of neurons in the brains of individuals born during this period (Spalding et al., 2005). On the basis of their analysis of postmortem brains from individuals born before or during the period of nuclear testing, they concluded that virtually all the neurons in the adult (60 year-old) human occipital cortex are generated during fetal development and last a person's lifetime.

Why do mammals generate new neurons only in the hippocampus and olfactory bulb? Frogs and fish have eyes that

BOX 3.1 Neural Crest Cells: The Great Explorers

Neural crest cells are the great explorers of the vertebrate body. The migration of these cells was first demonstrated by Detwiler (1937) by labeling the premigratory cells with vital dye and seeing dye-stained descendents moving throughout the body. Their neuronal and non-neuronal descendents can be found almost everywhere. No other cell type undergoes such extensive migration during development. As crest cells migrate, they become exposed to a variety of extrinsic factors that influence both their journey and their fate. But crest cells are intrinsically specified to become multipotent explorers. It is, as usual, the balance between these intrinsic tendencies and the environmental influences that determines where and what any particular descendent of the neural crest will become. This box gives a brief view of what is known about the mechanisms of neural crest migration.

To begin to migrate, crest cells must first leave their home port: the neuroepilium in which they arise. As the neural fold closes, crest progenitors, which were at the lateral borders of the neural plate, become situated in the dorsalmost part of the neural tube (Chapter 2). At this point they begin to behave differently from the rest of the cells in the neuroepithelium. They go through what is called an epithelial to mesenchymal transition (EMT) (**Figure 3.23**A) (Kuriyama et al., 2008). Mesenchymal cells are loosely packed nonspecialized cells, usually of mesodermal origin, that move around the body associated with connective tissue and extracellular matrix. Migrating neural crest cells share many of these mesenchymal properties. To leave the neuroepithelium, neural crest cells first lose their apical tight junctions to each other and to their neighbors. A major component of tight junctions is the protein Occludin. Occludin is dramatically downregulated in premigratory neural crest cells and as a result these begin to lose their apicobasal polarity and detach from their neighbors on either side. But they are not yet ready to let go of these neighbors. To do this they must also downregulate cell adhesion molecules. Neural Cadherin (N-Cad) is a homophilic cell adhesion molecule expressed on the membranes of all cells of the neural tube including premigratory crest cells. It works as a kind of tissue-specific glue. Interference with N-Cad function through expression of a dominant negative version of the protein causes these cells to lose contact with each other (Kintner, 1992). As we learned in Chapter 2, early crest cells are specified by the expression of the transcription factor genes *slug* and *snail*. The premigratory neural crest cells also express another transcription factor called *twist*. These transcription factors directly downregulate N-Cad and tight junctional proteins such as Occludin and so are important for the EMT of neural crest cells.

Once their tight junctions and homophilic adhesions with their neighbors have been lost, the premigratory crest cells face one more barrier before they can leave the confines of the neural tube. They must break through the heavy basal lamina composed of extracellular matrix proteins that completely surrounds the neural tube. To do this, they secrete special proteases, called matrix metalloproteases (MMPs). These proteases digest the extracellular matrix of the basal lamina, creating a hole for the crest cells to escape through so that they can begin their explorations of the rest of the body (Duong and Erickson, 2004).

The EMT of neural crest cells is in many ways similar to the first steps of metastasis in invasive cancers. Nonmetastatic tissues like the cells of the neuroepithelium have an apicobasal polarity with tight junctions and cadherins linking cells to their neighbors, keeping them in place. In early stages of metastatic cancer, tight junction proteins like Occludin and cadherins are downregulated. These cells may also then express MMPs and digest the basal lamina that is holding them in. Slug and snail expression are often upregulated in metastatic cancers. When metastatic cancer cells have made a full EMT, they are able to spread throughout the body, often via the blood stream, and invade other tissues. Of course, one of the key differences between normal neural crest cells and metastatic cancer cells is that crest cells are still restricted in terms of their proliferative potential, whereas cancer cells have somehow lost their growth control regulation.

Once the neural crest cells have left the neural tube, they begin to migrate. Large extracellular matrix molecules, such as collagen, laminin, and fibronectin, are known to support the migration of neural crest cells, for when these cells are dissociated from the embryo and plated onto tissue culture dishes coated with extracellular matrix, they migrate freely. The receptors for these extracellular matrix molecules, heterodimers of integrin proteins, are expressed by the migrating neural crest cells, and perturbation of these receptors inhibits neural

BOX 3.1 Neural Crest Cells: The Great Explorers—Cont'd

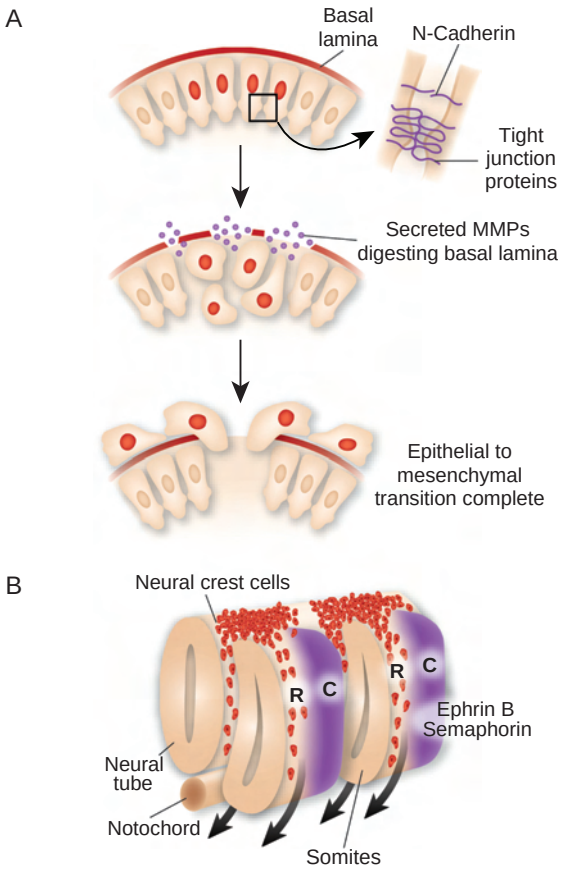

Fig. 3.23 Migration of the neural crest. A. The epithelial to mesenchymal transition (EMT). Crest cells (red nuclei) in top panel are situated in the dorsal neural tube and attached to each other and to their neighbors by tight junctions and N-cadeherin. In the middle panel, under the influence of the transcription factors Slug, Snail and Twist, crest cells downregulate tight junctional proteins such as ocludin, cell adhesion proteins such as N-cadherin, and begin to secrete MMPs to digest the overlying basal lamina of the neural tube. In the bottom panel, the crest cells complete the EMT by escaping through the hole digested in the basal lamina. B. Crest cells migrate in streams supported by extracellular matrix material, but expressing the receptors EphB and Neuropilin, they avoid the caudal half of each somite which expresses the repulsive ligands Ephrin B and Semaphorin for these receptors. C. Crest cells exhibit contact inhibition. Left. When two clumps of crest cells are cultured near each other, the cells migrating away from the centers of each clump do not mix when they encounter each other. Right. When a crest cell clump is cultured next to another tissue type such as mesoderm, the migrating crest cells do not avoid this tissue but rather migrate into and over it.

crest migration. If either β1-integrin, or its heterodimeric partner, α4-integrin, are blocked with specific antibodies, neural crest migration is blocked (Lallier et al., 1994; Kil et al., 1998).

In the cranial region, the cells migrate along the mandibular, hyoid, and branchial arches to their various regions in the head and neck. The neural crest from the trunk takes two basic routes from the neural tube: the ventral route, along which the cells that will form the sensory, enteric, and autonomic ganglia follow, and the dorsal or lateral route, in which the cells that will form the pigment cells in the epidermis predominate (Weston, 1963). A characteristic feature of emerging neural crest cells is that they migrate in interspersed streams. This is particularly clear in the trunk region where neural crest cells migrate through the rostral half of each somite but avoid the caudal half, which remains crest free (Figure 3.23B). What is responsible for these channeled migratory routes? The Eph receptors and Ephrin ligands were first identified for their roles in repulsive guidance of axonal growth (see Chapter 5). EphrinB is expressed by the caudal halves of the somites, while EphB the receptor for this repulsive ligand, is expressed by the migrating neural crest cells (Krull et al., 1997; Wang and Anderson, 1997). Semaphorins are also repulsive guidance molecules for axon growth (discussed in more detail in Chapter 5), and expressed in the caudal half of each somite. The Semaphorin receptor, Neuropilin, is expressed in the migrating crest cells. The same molecules are used to create crest-free zones in the cephalic region creating the streams of crest that flow between the branchial arches. These inhibitory guidance factors thus form the molecular riverbanks that break the flow of migrating crest cells into streams. *In vitro*, cells at the edges of neural crest clusters have active filopodia and migrate rapidly away from the center of the cluster, while the cells in the middle of the cluster have few filopodia and do not show such direction in their migration. This is because neural crest cells display contact inhibition of locomotion as demonstrated by the fact that when two migrating crest cells meet, they collapse their protrusions, transiently stop, and then migrate away from each other (Carmona-Fontaine et al., 2008). When a neural crest cell meets another cell type, such as a mesodermal cell, however, it does not show contact inhibition. Similarly, explants of neural crest cells do not invade each other, whereas cells migrating from a neural crest explant infiltrate explants of other cell types (Figure 3.23C). This homotypic contact inhibition of locomotion means that cells at the front of migration streams tend to move forward, and the lack of heterotypic contact inhibition means that when leading crest cells encounter a different type of tissue, they invade it. This behavior is again reminiscent of that shown by metastatic cancer cells.

The promotion of migration by extracellular matrix proteins, the guidance of these cells into streams by repellent molecules such as ephrins and semaphorins, and the contact inhibition that causes these cells to move away from each other and invade different kinds of tissues, should be sufficient to account for the far and wide migration patterns of neural crest cells. But on top of all this, there may also be positive, chemoattractive cues released from targets of neural crest migration, although there is as yet no convincing evidence for chemotaxis of crest cells up a gradient of such factors.

The route that crest cells take is to some degree determined by the environment in which they find themselves. For example, the neural crest from the most anterior part of the developing spinal cord migrates into the gut to form the enteric nervous system, while the neural crest from somewhat more caudal levels of the spinal cord never migrates in to the gut, but instead collects near the aorta and

Continued

BOX 3.1 Neural Crest Cells: The Great Explorers—Cont'd

forms the sympathetic ganglion chain. Transplantation of neural crest cells from anterior (enteric ganglion forming) levels of the embryo to more posterior regions results in the anterior crest cells following the posterior pathways and making sympathetic neurons instead of enteric neurons (Le Douarin et al., 1975; Le Douarin, 2004). As noted in Chapter 2, the neural tube has a considerable amount of pattern controlled in part by the regional expression of Hox transcription factors. The neural crest that migrates from the cranial regions of the neural tube also has positional identity, and this is also dependent on the Hox code. The figure shows the migration of the neural crest from the rhombomeres. The cranial crest contributes many cells to three branchial arches. The neural crest that migrates into these arches will give rise to most of the skeletal and cartilage of the skull and face. The unique contribution of the different regions of cranial neural crest has provided an opportunity to test for the specification of these cells and their migratory patterns. The crest cells from rhombomeres *r1* and *r2* migrate into the first (mandibular) arch, the crest from *r4* into the second (hyoid) arch, and the crest from *r6* and *r7* into the third (branchial) arch (Kontges and Lumsden, 1996). The crest in each of these arches differentiates into specific skeletal elements of the face or jaw (**Figure 3.24**). The neural crest from each rhombomere continues to express the same pattern of *Hox* genes as it migrates from the neural tube, and thus has a unique identity. This unique identity can be demonstrated by transplantation experiments where the crest from one rhombomere is transplanted to the region of another, and its migration and further development are monitored (Noden, 1975). Crest cells that would normally populate the third arch were excised and replaced with first arch crest cells. The transplanted crest cells migrated into the third arch, but instead of making neck cartilage, they formed beaklike projections from the neck and a complete, duplicate first arch skeletal system in their new location. Thus, it appears that the patterning of branchial arch skeletal and connective tissues is an intrinsic property of the cells of the neural crest prior to their emigration from the neural tube. Although they can use the same cues to migrate through the branchial arches, they will differentiate in accord with the Hox code specific for their position or origin.

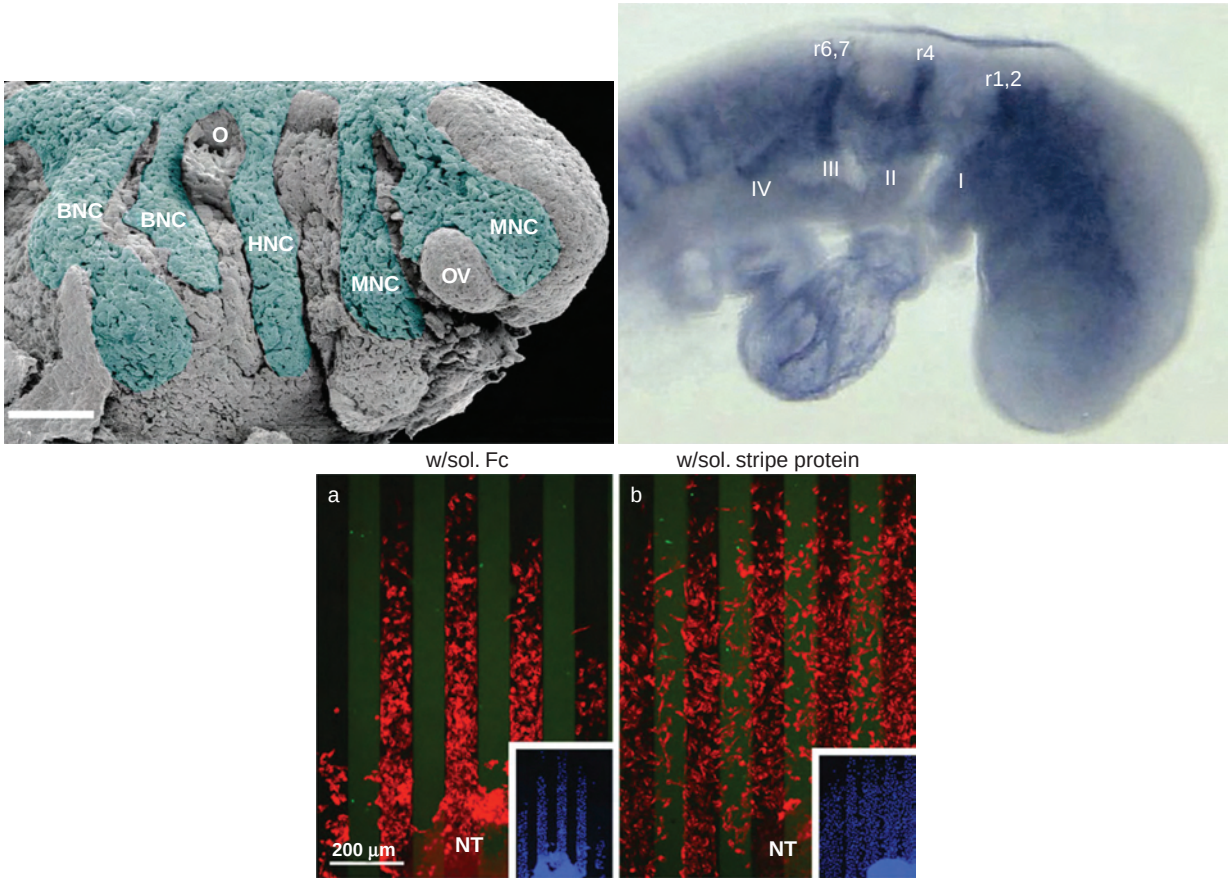

Fig. 3.24 Streams of neural crest. A. A scanning electron microscopic view of neural crest cells migrating in the first/mandibular (MNC), the second/hyoid (HNC), and the third and fourth branchial (BNC) arches in a salamander embryo. O = otic vesicle, OV = optic vesicle. B. A similar pattern is seen in a chick embryo stained with the neural crest specific marker HNK-1. Rhombomeres 1 and 2 (r1,2) contribute to the first arch, r4 contributes to the second arch and r6,7 contribute to the third and fourth arches. Left of that the most anterior of the segmental spinal streams can be seen. C. Crest cells in culture avoid stripes coated with Ephrin B (left), but if soulable EphrinB is added to the medium, this binds the EphB receptors on the crest cells and makes them insensitive to the coated stripes which they then no longer avoid. *(A from Falck et al., 2002; B and C from Mellott and Burke, 2008.)*

grow, birds learn a new song. What is the advantage to the mammal? Since both the olfactory bulb and the hippocampus are involved in the formation of olfactory memories, the neuronal turnover in these regions could be important in a seasonal change in nests or mates. Altman observed that the neurogenesis of the brain proceeds in two basic phases. The large projection neurons (or macroneurons) are generated early in embryonic development, while the smaller neurons (or microneurons) are generated later in development, through the postnatal period and even into adulthood. These later generated microneurons are then integrated into the framework provided by the macroneurons. Altman pictured this second stage of neurogenesis as a way for environmental influences to regulate the neurogenesis and produce a brain ideally suited to its environment. In one of the last reviews of his work, Altman (Altman and Das, 1965) summed up his hypothesis: "We postulate that this hierarchic construction process endows the brain with stability and rigidity as well as plasticity and flexibility." Although it has been difficult to prove that persistent neurogenesis in a particular region of the brain is necessary for behavioral plasticity in that brain region, recent studies are consistent with Altman's hypothesis. Selective deletion of the newly generated neurons in the SVZ does lead to some functional deficits, particularly with olfactory memory tests (Imayoshi et al., 2008). Imayoshi et al. used a tamoxifen inducible Cre-recombinase strategy similar to that described above, except in addition to labeling the progeny of the adult neural progenitor cells, they mated the inducible Cre mice to mice engineered with a Cre-activated toxin. This effectively killed all the progeny of the adult neural progenitor cells, and led to a decline in the total number of granule cells in the olfactory bulb over time. In the hippocampus, the addition of new neurons was also blocked in these mice. Although the mice could no longer make new neurons, they did retain the ability to make new *olfactory* memories; however, the mice showed a significant impairment in their ability to make new hippocampal-dependent *spatial* memories. This elegant study demonstrates the importance of adult neurogenesis, at least in the hippocampus, and suggests that when you learn directions to a new place, you have neurogenesis to thank.

SUMMARY

The enormous numbers of neurons and glia in the brain are generated by progenitor cells of the neural tube and brain vesicles. The progenitor cells from the early embryonic nervous system undergo many symmetric cell divisions to make more progenitor cells, while the progenitor cells in the late embryo are more likely to undergo an asymmetric division to generate neurons and glia. The production of both neurons and glia from the progenitor cells is under tight molecular control, and this allows the proper numbers of both neurons and glia to be produced for the proper functioning of the brain. Interactions between the neurons and the progenitor cells regulate their proliferation in both positive and inhibitory ways. Overall, a remarkable coordination takes place to regulate proliferation in the nervous system during development, and mutations in specific genetic pathways involved in neurogenesis can lead to childhood tumors and gliomas in adults. Once the developmental period of neurogenesis is complete, most areas of the brain do not generate new neurons, even after damage. This has led to the concept that you are born with all the neurons that you are going to ever have. However, in recent years, it has become clear that certain regions of the brain, the hippocampus and the olfactory bulb, continue to add new neurons throughout life. This continual addition of neurons in these regions may allow for greater plasticity in these specific brain circuits.

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