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Selected Comparison of Immune and Nervous System Development

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I. Introduction

The developing mammalian nervous system shares many similarities with the formation of the immune system. Many of these similarities are at the level of homology or analogy, such as the concept of "memory," but others extend into actual identity, as is the case for a number of different molecules, possibly reflecting their use in related functions. The purpose of this chapter is to review neural development for an immunological audience, with an aim of selecting a few comparative examples in which immunological information may provide insight into neurobiological developmental mechanisms, and perhaps vice versa. Emphasis is placed on neurogenesis within the developing mammalian cerebral cortex, as compared to immunological events in the thymus. Two aspects of current interest receive particular attention here: programmed cell death (PCD, or apoptosis) and the hypothesis that some form of DNA rearrangement occurs within the nervous system, especially in light of recent studies on nonhomologous end-joining (NHEJ) molecules. In each major section, a neurobiological feature is discussed, and germane aspects of immunological development are also considered.

II. Major Cellular Components of the Nervous System

The primary constituents of the mature nervous system are neurons and glia. In addition, there are a large number of other cell types that contribute to normal brain function, such as endothelial cells, meningeal cells that form an outer covering of the brain, and choroid plexus cells that produce cerebrospinal fluid, which are not discussed further here. Still other cell types also exist during development of the brain which appear transiently, such as cells from macrophage lineages that have phagocytic functions. In considering the cellular constituents of the brain, it is important to note the developmental age, since the complement of cells varies for particular developmental stages.

A. Effector Cells: Neurons

Neurons come in a bewilderingly wide range of sizes and shapes (Stevens, 1979). These differences were first noted in the late nineteenth century by

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early neuroanatomists/histologists such as Golgi and Ramón y Cajal, who began to categorize neuronal types based on their histological appearance (Ramón y Cajal, 1901, 1960). Indeed, the Golgi technique of silver precipitation to visualize single neurons, along with many neuronal descriptions, such as "granule" and "pyramidal" neurons, are in use to this day; many modern techniques, such as filling live neurons with fluorescent dyes, have both confirmed and augmented the concept that neuronal morphologies are complex. Neurons are the primary effector cells of the nervous system by way of their electrical excitability and synaptic contacts. By definition, neurons are postmitotic, and it is of note that despite their expression of a range of potentially oncogenic molecules, mature neurons remain postmitotic, as further evidenced by the lack of central nervous system (CNS) neoplasms that contain pure populations of neurons.

Related to their morphological differences, neurons also have distinct anatomical connectivities, physiological properties, neurotransmitter identities, and probable differences in a variety of other molecules, such as the protocadherins (Hirano *et al.*, 1999; Kohmura *et al.*, 1998; Obata *et al.*, 1995, 1998; Sago *et al.*, 1995; Wu and Maniatis, 1999). Anatomical differences in neurons are not intuitive from a molecular immunological perspective, since there are no specific molecular markers such as T cell receptors (TCRs) or immunoglobulins (Ig's) to mark these differences. However, the actual location of a single neuron in three-dimensional space contributes to its virtually unique identity. For example, visual space is mapped precisely and topographically onto the cerebral cortex (Shatz, 1987; Shatz and Luskin, 1986), and the neuron representing this point in space, along with its shape and physiology, has a unique identity. To maintain a high degree of organization necessary to interpret the visual world, mature neuron cell bodies are not motile, and their synaptic contacts are relatively stable. However, there can be remodeling of the system at the level of synaptic contacts (Mooney *et al.*, 1996), a phenomenon that may account for synaptic "plasticity" (Katz and Shatz, 1996; Shatz, 1990b, 1996), which includes features of learning. It is also worth noting that a typical neuron does not simply receive a single input from a single cell, as is often represented diagrammatically for didactic purposes. In fact, a single CNS neuron can receive on the order of 10,000 synapses and be connected to 1000 other neurons (Stevens, 1979). The importance of these attributes for this discussion is that neurons are, in reality, extremely heterogeneous (some would argue unique from neuron to neuron), stationary cells that are further capable of synaptic plasticity as well as connected in complex manners to many other neurons. Considering that the human brain contains on the order of 10^{11} neurons with the aforementioned connectivities and phenotypic differences, the brain achieves a degree of complexity that compares favorably with that calculated for Ig's and TCRs.

The effector cells of the immune system, lymphocytes, are quite different compared to neurons. In particular, the primary effector functions of the immune

system involve interactions between antigen and antigen receptors of highly mobile cells that maintain the ability to proliferate through molecularly defined developmental stages (Darnell *et al.*, 1986). The existence, for example, of B cell tumors of pro–B, pre–B, and mature B cell lineages underscores this capability (Whitlock *et al.*, 1985). In addition, cell–cell communication is not required for terminal stages of effector function, as seen by the role of soluble Ig's. From this comparison, the mature effector cells of the nervous system—neurons—are really very different from T cells and B cells in many respects. What stands out in this comparison is the multifaceted complexity of neurons that, in degree, is similar to the molecular complexity of lymphocytes achieved through the formation of antigen receptors.

B. Noneffector Cells: Glia

The other major cell type in the brain, glia, can be distinguished morphologically, biochemically, and molecularly from neurons and are traditionally viewed as supporting cells for neurons (Mission *et al.*, 1991; Rakic, 1988). It is estimated that there are nearly 10 times more glia than neurons within the CNS (Truex and Carpenter, 1971). Two general types of glia are the astrocytes (Truex and Carpenter, 1971), which are often closely apposed to blood vessels, and myelin-producing oligodendrocytes (Raine, 1997), or the peripheral nervous system (PNS) counterpart of oligodendrocytes, the Schwann cells (Brockes *et al.*, 1979; Weiner and Chun, 1999). In general, glia are not in synaptic contact with neurons and thus do not participate directly in the effector functions of neurons. Glial cells maintain the ability to proliferate. Astrocytes (Alberts *et al.*, 1983; Brockes *et al.*, 1979) express a marker protein called glial fibrillary acidic protein that can also be expressed by an embryonic counterpart known as radial glia (Rakic *et al.*, 1974; Woodhams *et al.*, 1981). In addition to these classical glia, a third type of glia are microglia, which are actually derived from a macrophage lineage (Hiremath *et al.*, 1998; Truex and Carpenter, 1971). Perhaps the clearest similarity of glia to immunological cells are those involved in antigen presentation in the immune system. It is likely that an extensive number of mechanisms utilized by antigen-presenting cells are maintained in nervous system microglia, including the involvement of cytokines and growth factors (Otero and Merrill, 1994). Moreover, there is evidence that some astroctytes are themselves capable of *in vivo* antigen presentation (Williams *et al.*, 1995).

III. Embryonic Divisions of the Nervous System

A. Neural Tube and Neural Crest

Neurons and glia of the nervous system arise from two classical subdivisions of the nervous system that reflect distinctions in their embryological origin (Cowan, 1979; Langman, 1981). This distinction arises around the time of neural tube

closure, at which point cells of the neural tube give rise to the CNS, whereas cells arising from the excluded lip of the closing tube—the neural crest—give rise to the PNS (Fig. 1).

The nervous system takes form at a point preceding formation of the primitive streak. In humans, this occurs by embryonic day 18 (E18); in the mouse (gestation about 20 days), this occurs around E7. The presence of the primitive streak in the dorsocaudal (tail) portion of the embryo marks the morphological beginning of the cephalad (head) location of the neural plate, which is simply a thickening of the proliferating ectoderm. Within hours in mice (days in humans), neurulation commences through inductive effects of the underlying mesoderm. The morphological result of induction is the formation of the neural groove that arises by cell proliferation in the lateral edges of the neural plate. The increase in cells laterally creates neural folds that bend toward one another and eventually fuse at the midline to form two important structures: the neural tube, which gives rise to the CNS proper, and the neural crest, which gives rise to the PNS (Fig. 1), along with a multitude of other nonneural tissues, discussed in the following section.

B. Peripheral Nervous System

The cells of the neural crest migrate to a variety of locations throughout the embryo to form the PNS, along with melanocytes; facial tissues, including odontoblasts and cartilage cells; some of the meninges (coverings of the brain); and other tissues as well (LeDouarin, 1980). By comprising the PNS, the neural crest gives rise to the dorsal root neurons of the dorsal root or sensory ganglia, Schwann cells that are responsible for myelination of peripheral axons, the sympathetic and parasympathetic ganglia (or parts of the autonomic nervous system), and the chromaffin cells of the adrenal medulla. Two remarkable features of the neural crest are their pluripotency—giving rise to a variety of tissues—and their capacity to migrate into the surrounding mesoderm.

An additional group of neurogenic regions that are embryogically distinct from the neural crest and the CNS are referred to as placodes (from the Greek *plate-form*), such as the olfactory placode. These regions appear to be most closely akin to the neural crest in being distinct from the neural tube and possessing cells with substantial migratory capacity, such as those giving rise to neurons producing gonadotropin-releasing hormone. The remainder of this chapter focuses on the CNS; however, it should be noted that the overwhelming majority of studies on "neuronal" cell lines in the literature are of most relevance to the PNS, where the overwhelming majority of such cell lines (e.g., neuroblastomas) arise.

C. Central Nervous System

With the formation of the neural tube, cell proliferation (hyperplasia) and increases in cell size (hypertrophy) produce a decidedly asymmetrical and enlarging

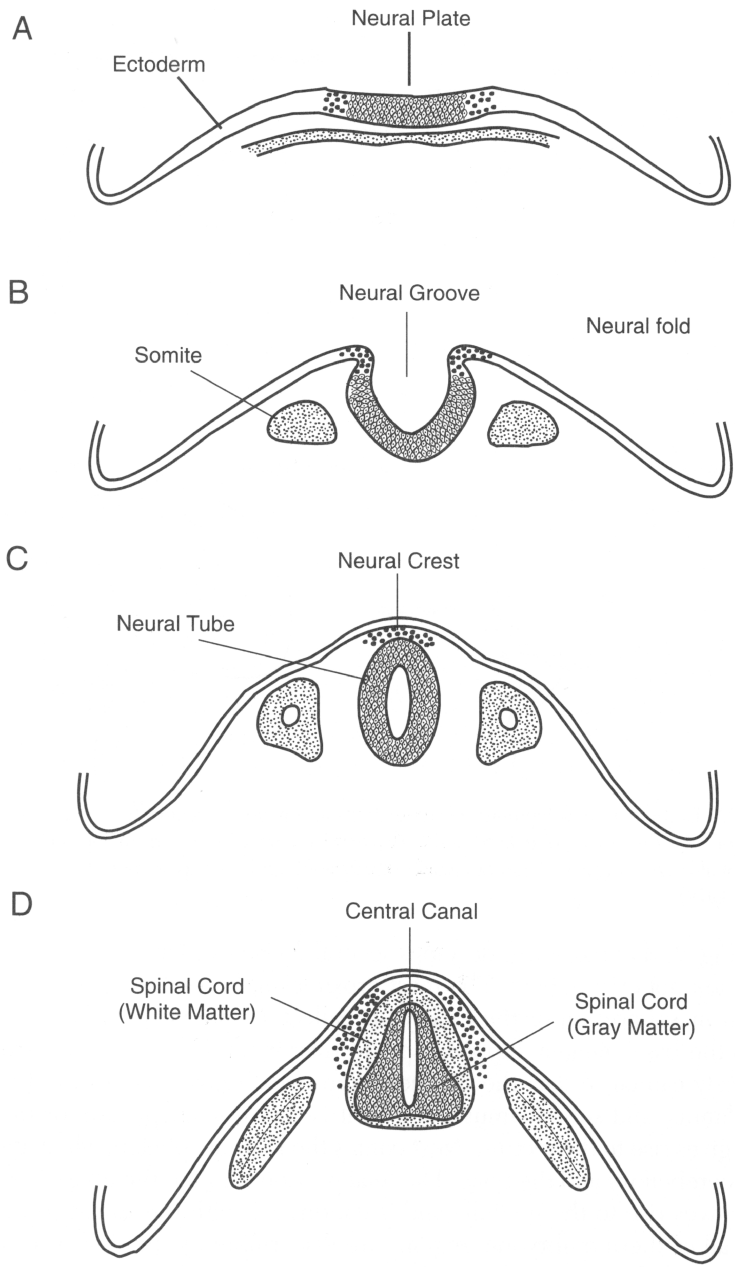

FIG. 1. Embryonic derivation of the peripheral nervous system (PNS) and central nervous system (CNS). Transverse section through an early embryo reveals the neural crest cells, which migrate away and give rise to the PNS. In contrast, the CNS derives from closure of the neural groove to produce the neural tube. Adapted from Cowan (1979).

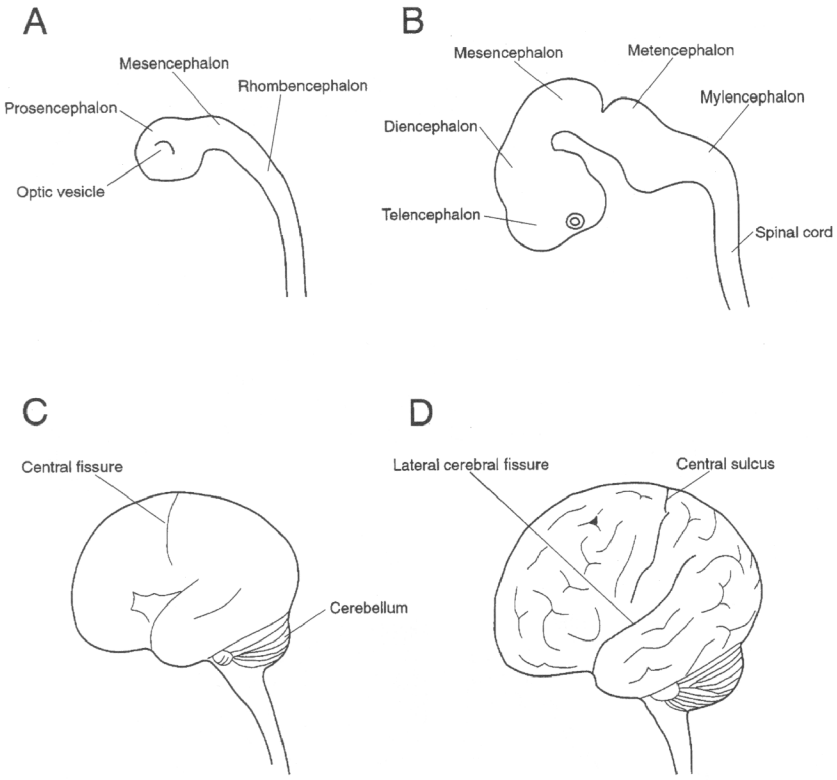

FIG. 2. Embryonic development, nomenclature, and general organization of the neural tube. Names of tube divisions are as indicated. The cerebral cortex derives from the prosencephalon/telencephalon, growing in cell number and size to form its adult-like appearance (D). Adapted from Cowan (1979).

tube (Fig. 2) that moreover develops several distinctive kinks and bends, whose names are not critical here. The most proximal and eventually predominant (at least in mass) portion of the embryo is called the telencephalon, which gives rise to the basal ganglia, hippocampal formation, and the cerebral cortex. Immediately caudal to it is the diencephalon, which gives rise to the thalamus, epithalamus, and subthalamus. Next to it is the mesencephalon, or midbrain, which gives rise to the tectum. Next comes the metencephalon, which gives rise to the cerebellum and the pons. The final portion is called the myelencephalon, which gives rise to the medulla and is continuous with the spinal cord. In humans, all of these embryonic regions are discernible by the first trimester (by about E12 in the mouse).

Contrasting with the nervous system, the immune system arises embryologically from extraembryonic stem cells that are more generally part of hemopoiesis, occurring through 10 weeks' gestation in humans (E12 in the mouse)

(Cooper and Nisbet-Brown, 1993). During this period, colonization of endoderm derivatives for T cells and B cells takes place. T cell precursors colonize the thymic anlage (primordium) around E10–E11 in the mouse, the anlage forming from the third and fourth pharyngeal pouches. A similar time course of colonization occurs for B cell precursors; however, they populate the fetal liver, an endodermal outgrowth of the embryonic hepatic diverticulum. By E15, they are also found in the spleen, and before birth, can be found in their adult-like location, the bone marrow. The embryonic origin and developmental compartments for both T cells and B cells are completely distinct compared to the nervous system that arises from the embryonic ectoderm.

The majority of the nervous system originates from the telencephalon: it will eventually comprise ~85% of the total mass of the brain (Truex and Carpenter, 1971). The focus on its major component, the cerebral cortex, thus serves both as an illustrative example and as a paradigm for most of the CNS in exploring developmental issues.

IV. Embryonic Development of the Cerebral Cortex

A. Organization

The embryonic cortex arises from the proximal portion of the closed neural tube (first called the prosencephalon, Fig. 2), which later becomes the telencephalon mentioned above. At its earliest stages, it consists of a single layer of pseudostratified epithelium (Fig. 3) that forms a single sphere, then later as the paired hemispheres that give rise to the forebrain. By examining a magnified cross-section of the telencephalic wall, several obvious parts can be distinguished using simple, histological stains.

The inner portion of the epithelium (toward the bottom of Fig. 3) that is in contact with the developing ventricles is aptly referred to as the ventricular zone (VZ). This is the zone of cell proliferation from which the vast majority of all neurons and glia of the cerebral cortex are derived. Somewhat nonintuitively, this region historically is considered apical. The outer, or basal, surface of the brain is also referred to as the pial surface because it is surrounded by the pia mater, consisting of supporting cells of neither neuronal nor glial lineage (probably derived from the neural crest). Cells in the epithelium, at least while the layers of cells in the epithelium are few, appear to have attachments to both the pial and ventricular surface. While the VZ remains present throughout neurogenesis, the histologically distinguishable layers, as seen in a tissue section extending from the pial to the ventricular surface, change with time (Fig. 4). This change reflects the generation of more superficial embryonic cell layers that are formed by the production and migration of postmitotic neurons and by the ingrowth and outgrowth of axons. The

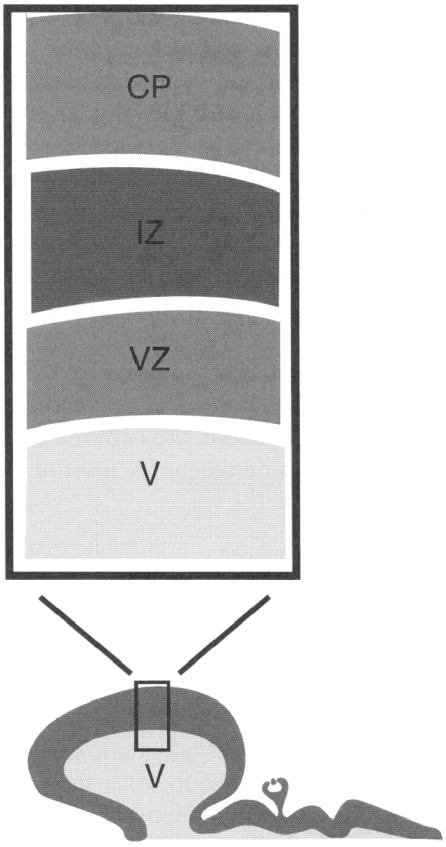

FIG. 3. Embryonic zones in the developing cerebral cortex. A section from the cerebral wall is enlarged to reveal the three major embryonic zones that are visible by standard histology. The ventricle (V) is surrounded by the ventricular zone (VZ), the zone of cell proliferation during neurogenesis. The intermediate zone (IZ) sits superficial to the VZ and contains growing processes and migrating postmitotic neurons. The cortical plate (CP) contains differentiating postmitotic neurons that will remain in the adult as the cortical gray matter. The microscopic components in these zones are shown in Fig. 4.

latter process, combined with cell migration, produces a region just superficial to the VZ, which is aptly named the intermediate zone (IZ). Just superficial to the IZ is the cortical plate (CP), which consists of postmitotic neurons that have reached their adult-like location. Only the CP persists into adulthood, transformed into the six-layered cerebral cortex. The VZ is essentially lost, while the IZ becomes a zone of axon fibers commonly referred to as white matter.

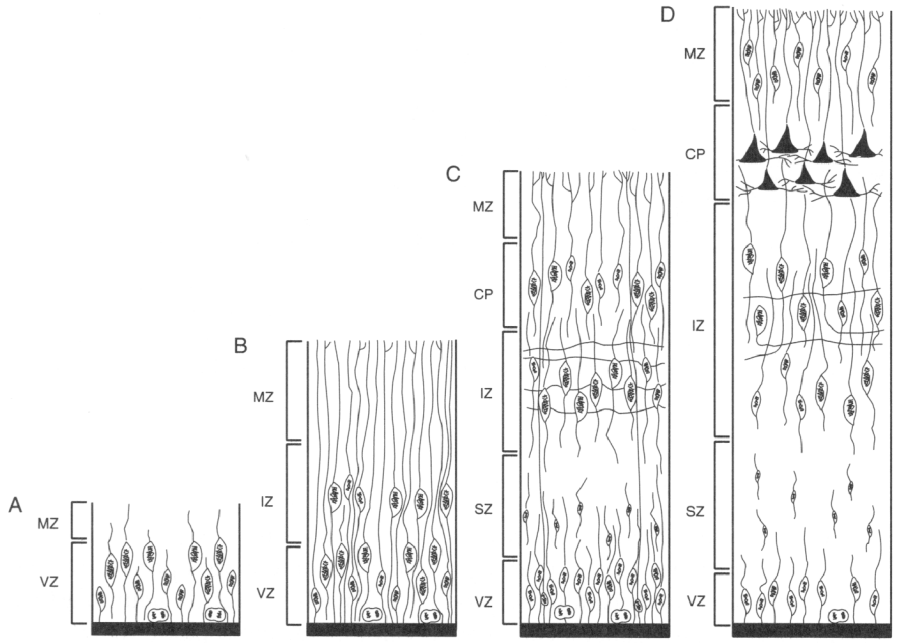

FIG. 4. Microscopic anatomy of the embryonic cortical zones. These change with time as development proceeds (increasing age from left to right). (A) The early proliferative stage consists of a pseudostratified epithelium. (B and C) With development, the cerebral wall increases in width. Some cells, with processes stretching from the ventricular surface (black bar) to the pial surface (top of each illustration) represent radial glia that are used by some young neurons during migration to the pial surface. (C and D) With further development, more mature neurons (black triangular shapes) begin to differentiate superficially in the cortical plate (CP), along with the ingrowth/outgrowth of axons in the intermediate zone (IZ; lines parallel to the black bars). MZ, Marginal zone; SZ, subventricular zone; VZ, ventricular zone. Adapted from Cowan (1979).

Once located within the postmitotic CP, a prominent feature of cortical development can be seen: the formation of cellular layers based on the "birthdate" of a neuron. Newly postmitotic neurons do not assemble randomly within the CP, but assume histologically visible layers. The normal adult cerebral cortex has six layers, and cells within each layer have distinguishable morphologies and anatomical projections. In addition, the neurons within a layer are not generated randomly but instead form in an inside-out manner. Thus, the oldest cells are located closest to the ventricle, while the younger cells must migrate past the older ones to take a more superficial position (closer to the pial surface). In this way, the neurons of the cerebral cortex form cellular layers on the basis of their birthdate, in addition to layers that have both functional and anatomical specificity.

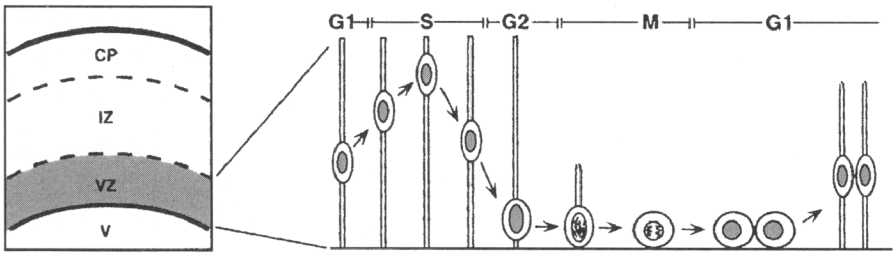

FIG. 5. Interkinetic nuclear migration of neuroblasts within the ventricular zone (VZ). Proliferating neuroblasts undergo characteristic shape changes linked to the cell cycle. After mitosis, the daughter cells can either continue proliferating, repeating this process, or can become postmitotic and migrate superficially to differentiate in the cortical plate (CP) (see Figs. 3 and 4). IZ, intermediate zone; V, ventricle.

B. NEUROGENESIS

Within the proliferative VZ, two cellular events must occur. First, a sufficient number of progenitors must be produced over a limited period. Second, postmitotic neurons must also be produced. To achieve this, a neuroblast can assume one of three general cell fates. It can reenter the S phase to again undergo mitosis, can become permanently postmitotic, or can undergo PCD. The former two fates constitute the historical view of how neurons of the cortex form and are examined here first, with recent data on cell death treated in more detail below. A characteristic of cell proliferation that is associated with neurogenesis is termed interkinetic nuclear migration (Fig. 5), in which the shapes of neuroblasts change in concert with phases of the cell cycle (Sauer, 1935; Seymour and Berry, 1975).

As a given cell undergoes DNA synthesis (Angevine and Sidman, 1961; Sidman and Rakic, 1973; Sidman *et al.*, 1959), its nucleus is positioned above (basal to) the ventricular surface, while the cell itself has a fusiform morphology. Following the S phase, the nucleus moves apically while retracting its basal process until reaching the ventricular surface. This results in a rounding up of the cell body, whereupon it undergoes mitosis. With the completion of mitosis, the neuroblast regains a fusiform morphology to continue proliferation to maintain or expand the blast population, depending on the stage of development. The resulting two daughter cells (Zhong *et al.*, 1996) produced by mitotis can, alternatively, become postmitotic, migrating in a basal direction to locate just beneath the pial surface to form the future cerebral cortical gray matter, which at this age is the CP. The outlines of this phenomenon have been known since the 1930s; however, the significance of it for neurogenesis remains obscure (Chun, 1999).

A problem faced by migrating neurons is that they must find their way from the VZ to their more superficial location. This problem is exacerbated as

development proceeds because of the increasing width of the cortical wall (i.e., increasing ventricle-to-pial distance) caused by the accumulation of postmitotic neurons and the development of afferent and efferent fiber systems (see below). One mechanism that seems to be used by migrating neurons is their migration to the pial surface along a special kind of glial cell (which is also generated from the VZ). These radial glia (Culican *et al.*, 1990; Rakic *et al.*, 1974; Woodhams *et al.*, 1981) (e.g., Fig. 4C) span the long distances between pia and ventricle and appear to serve at least partially as a substrate along which the neurons can migrate. However, nonradial migration also clearly occurs during formation of the cortex, as do neuronal contributions from noncortical areas. Thus, to reach its final location, a newly postmitotic neuron must interact extensively with an array of different cells and cell types.

The primary technique that has allowed the demonstration of these neurogenic phenomena is use of labeled nucleotide analogues that are incorporated into DNA during the S phase of the cell cycle within the VZ, combined with visualization of cells in tissue sections using histology. Classical techniques that were used in the 1950s and continue to be used today utilize [^{3}H] thymidine incorporation followed by tissue autoradiography (Angevine and Sidman, 1961; Berry and Rogers, 1965; Caviness and Sidman, 1973; Chun and Shatz, 1988, 1989a,b; Sidman *et al.*, 1959). Labeled cells can be detected using photographic emulsions overlaid on tissue sections. After photographic developing, location of ^{3}H-labeled β-particle emission is marked by silver grain deposition over a labeled cell and can be seen using bright- or dark-field microscopy. More common today is the use of bromodeoxyuridine (BrdU) that is incorporated like thymidine and that can be visualized using immunohistochemistry (Blaschke *et al.*, 1996, 1998; Pompeiano *et al.*, 2000; Takahashi *et al.*, 1995; Weiner and Chun, 1997b, 1999).

The neurogenic formation of the cortex has parallels with T cell development in the thymus (Weissman, 1967, 1973). The nomenclature for the thymus as compared to the CNS is potentially confusing, since the less mature T cells are present in the thymic "cortex." Like cells in the CNS VZ, cells in this outer thymic region can be labeled by brief pulses of nucleotide analogues, following which labeled cells can be studied (e.g., by tissue autoradiography or by BrdU immunohistochemistry). The outermost portion of the thymic cortex, a rim of cells on the order of eight cell diameters, is similar to the proliferative VZ. Using [^{3}H]-thymidine, what are now known to be $CD4^+CD8^+$ double-positive cortical thymocytes were shown to be "born" in this discrete zone, whereupon they migrate centripetally to the mature location of single-positive thymic T cells, the medulla. Unlike most of the CNS, proliferation and maturation of T cells represent a continual process that occurs throughout most of life. However, the existence of CNS stem cells (Gage *et al.*, 1995) raises the interesting possibility that related mechanisms may function at other developmental stages, if on a reduced scale.

C. Initial Process Outgrowth

A hallmark of the CNS is its precise connections among the appropriate populations of neurons (Shatz, 1987, 1996; Shatz *et al.*, 1988). To allow the development of these connections, two criteria must be met: (i) the growing axons must find the general target population, and (ii) a fine-tuning of the connection must be made so that the axons can synapse on the precise target cell. The first criterion appears to be met, at least in part, by the growth of young axons that interact with chemoattractants and chemorepulsive molecules (Dreyer *et al.*, 1967; Tessier-Lavigne, 1992). This occurs along specified routes that consist of extracellular matrix or cell surface molecules (Goodman, 1994; Goodman and Shatz, 1993; Grenningloh *et al.*, 1990). Some of these molecules, such as fibronectin, laminin, and proteoglycans as well as Ig superfamily members, probably involve the creation of a permissive (or nonpermissive) surface on which axons can travel, something like a highway that allows cars to travel but imparts little information about direction. Other molecules may serve to impart information—continuing the analogy, like a road sign or stoplight. By this kind of growth, axons can reach their target populations; this large body of information has been the subject of numerous excellent reviews (Goodman, 1994; Tessier-Lavigne, 1995; Tessier-Lavigne and Zipursky, 1998).

The second task is making the exact synaptic connection to the appropriate target cell. A popular model postulates that the "activity" of the axons—their propagation of action potentials and resultant synaptic function—allows axons to make these appropriate connections (Penn and Shatz, 1999; Shatz, 1990a). Evidence in favor of this hypothesis comes in part from studies using tetrodotoxin (a sodium channel blocker) to block activity. When this is done for the retinogeniculate pathway (from the retina to the thalamus), axons fail to sort out into their appropriate target layers, although the axons do reach the general target population and neurons appear to be normally organized. A recent report, however, complicates this view. Genetic deletion of Munc 18-1, which is required for neurotransmitter release from synaptic vesicles, results in a remarkably normal organization of the embryonic CNS (Verhage *et al.*, 2000), suggesting that at least this form of activity is nonessential for normal development. Key mechanisms for forming initial groupings of cells and initial connections remain an active area of discovery. One intriguing possibility is a role for a large gene family encoding cell surface molecules, the protocadherins, in the formation of synaptic connections (Kohmura *et al.*, 1998). These molecules likely have cell–cell recognition functions like the cadherins do (Takeichi, 1991; Takeichi *et al.*, 1990a,b), which may be consistent with a more elaborate system of cell–cell interactions in organizing the early nervous system.

By comparison, the immune system does not require precise connections between cells, nor do nervous system synapses function in cell–cell recognition. There do exist a plethora of Ig superfamily molecules with obvious relevance to

both immune and nervous systems (Williams and Barclay, 1988). Indeed, some are expressed or are present within both systems, such as Thy-1 (Brockes *et al.*, 1979; Lancki *et al.*, 1995; Williams and Barclay, 1988), cadherins (Muller *et al.*, 1997; Takeichi, 1991), major histocompatibility complex (Corriveau *et al.*, 1998; Germain, 1994), Ig's (Schatz, 1997; Weiner and Chun, 1997a), or signaling molecules such as *fyn* (Yasunaga *et al.*, 1996) that interact with protocadherins (Kohmura *et al.*, 1998). Whether there is truly shared functionality for these molecules that is common to the immune and nervous systems remains an open question, but whatever the case, the cellular substrates (e.g., synapses on postmitotic neurons for the nervous system) appear to be quite distinct.

V. Ventricular Zone Neuroblast Programmed Cell Death

PCD is a feature common to all multicellular organisms. It has a well-characterized and essential role in the immune system, perhaps best exemplified by cell selection of appropriate T cells within the thymic cortex. Estimates on the extent of PCD in the thymus are in the range of 97% (Egerton *et al.*, 1990; Shortman and Scollay, 1994; Shortman *et al.*, 1990). The major mechanism for this intrathymic death within the thymic cortex appears to be by "neglect" because of a failure of T cells to undergo positive selection (Surh and Sprent, 1994), whereas "negative selection" constitutes a comparatively minor form that takes place in the thymic medulla. An interesting historical note is that controversy existed for decades following the proposal that intrathymic cell death actually took place (Metcalf, 1966), and the lack of death was championed by anatomists, who noted the absence of histological evidence for intrathymic cell death (Poste and Olson, 1973). The vast majority of normal PCD in the thymus takes place within the thymic cortex, overlapping with regions of thymic cell production, based on S-phase incorporation of labeled nucleotides such as [^{3}H] thymidine (Surh and Sprent, 1994; Weissman, 1973). It is further notable that data obtained using [^{3}H] thymidine was a mainstay of many studies supporting the conclusion that PCD did not take place in the thymus (discussed in Shortman and Scollay, 1985, 1994; Shortman *et al.*, 1990). It was not until the use of terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) (Gavrieli *et al.*, 1992), which identified free DNA ends associated with PCD, that intrathymic cell death was demonstrated (Surh and Sprent, 1994).

What is the role of PCD in the developing CNS? Pioneering studies had identified cell death as a significant mechanism in matching neuronal number to target tissues (Hamburger, 1975; Hamburger and Levi-Montalcini, 1949; Hamburger and Oppenheim, 1982), and this form of PCD occurred among postmitotic neurons, likely utilizing mechanisms of synaptic competition and target-derived growth factors (Davies and Lumsden, 1984). This type of cell death appears to occur throughout the nervous system (Chun and Shatz, 1989a,b; Chun *et al.*,

1987; Verhage *et al.*, 2000). However, the theoretical existence of earlier developmental phases of neuroblast cell death in the VZ was largely ignored or dismissed (discussed in Blaschke *et al.*, 1996). Indeed, elaborate mathematical models have been constructed that must assume there is no PCD during neurogenic phases of development (Caviness *et al.*, 1995). Contrasting with postmitotic neurons, VZ neuroblasts have more similarities with immunological cells such as double-positive T cells within the thymic cortex. Neuroblasts do not have synaptic contacts at this stage of development, can proliferate, migrate, and have comparatively simple morphologies. Another common feature shared by cortical thymocytes (Turka *et al.*, 1991) and VZ neuroblasts (Chun *et al.*, 1991) is expression of recombination-activating gene 1 (RAG1; discussed further below), although the role of RAG1 in the nervous system remains obscure. These superficial similarities between thymocyte development and VZ neurogenesis led us to ask whether, like the thymus, PCD operated in the embryonic cerebral cortex.

To address this possibility, two techniques were developed and tested on normal and induced PCD using a variety of tissue and cell systems, including the thymus. *In situ* end-labeling plus ($ISEL^+$) (Blaschke *et al.*, 1996, 1998; Chun and Blaschke, 1997; Pompeiano *et al.*, 1998, 2000; Staley *et al.*, 1997; Zhu and Chun, 1998) is a much more sensitive variation of what was used to demonstrate intrathymic cell death, TUNEL. The second independent technique developed is based on ligation-mediated polymerase chain reaction (PCR) of blunt, 5′-phosphorylated DNA ends that are produced during apoptosis in the form of nucleosomal ladders that can be specifically amplified from apoptotic cells or tissues, including the normal thymus (Blaschke *et al.*, 1996, 1998; Chun and Blaschke, 1997; Staley *et al.*, 1997; Zhu and Chun, 1998). As an aside, these ends are identical to those generated by RAG cleavage (Schlissel *et al.*, 1993). Both techniques produced results that supported or extended PCD results obtained in the thymus using TUNEL, as well as in other tissues. Examples of thymic labeling in dexamethasone-treated (induced PCD) or normal thymus are shown in Fig. 6.

Notable in these figures is an increase in the number of dying cortical thymocytes compared to results obtained with TUNEL (Surh and Sprent, 1994), consistent with the ≈10-fold greater sensitivity of $ISEL^+$ as compared to TUNEL (Chun, 1998; Chun and Blaschke, 1997) and in closer agreement to previous estimates of cortical T cell PCD (Egerton *et al.*, 1990; Scollay and Shortman, 1985; Shortman and Scollay, 1985, 1994; Shortman *et al.*, 1990).

An example of what is observed in the embryonic cerebral cortex using $ISEL^+$ is shown in Fig. 7. Consistent with PCD identified in the thymic cortex, dying cells were present in the expected postmitotic neuronal compartments (IZ and CP). Most strikingly, PCD was also present in the VZ, the region of cell proliferation. This provided the first evidence for VZ neuroblast PCD, comparable to the evidence that was used to demonstrate intrathymic PCD (Surh and Sprent, 1994).

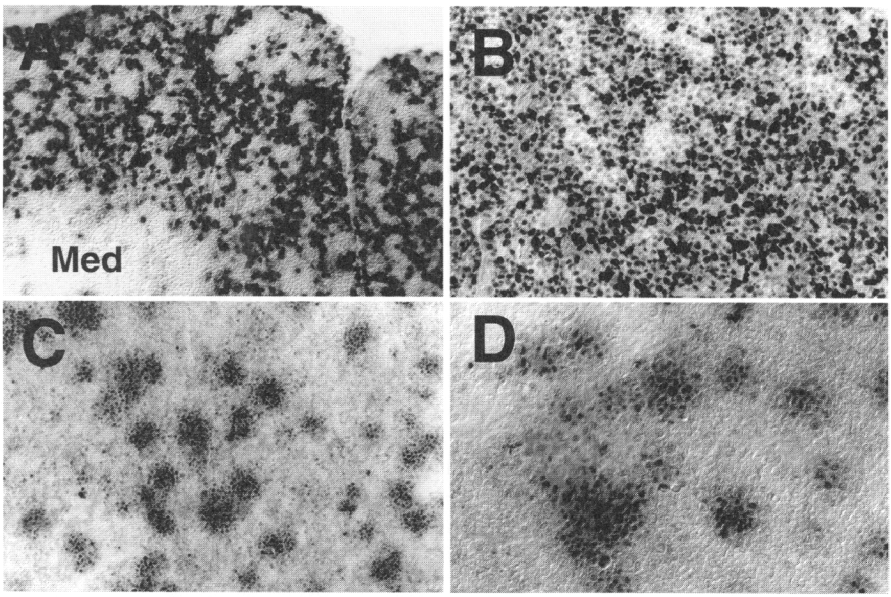

FIG. 6. ISEL$^+$ (*in situ* end-labeling plus) identifies induced and normal cell death in the thymus. (A and B) Dexamethasone-induced programmed cell death in the thymic cortex (low magnification in A, higher magnification in B). (C and D) Normal thymic cell death identified in the thymic cortex. Med, Medulla.

As already noted above, the concept of intrathymic PCD was met with strong denial by histologists, and an essentially identical response, now repeated some 15–20 years later, has occurred for VZ PCD because, here too, there is a lack of histologically identifiable cell death. The caveats associated with deriving conclusions based purely on histology and the use of nucleotide incorporation are obvious, based on past studies of the thymus. Indeed, histological techniques do not accurately identify PCD that is known to occur in other model systems, such as the extreme example of the small intestinal villus, where an entire villus population of cells dies and is replaced every few days (Pompeiano *et al.*, 1998).

The demonstration of significant PCD among VZ neuroblasts by ISEL$^+$ and ligation-mediated PCR has been independently supported by mouse null mutations in several pro–cell death genes. A major prediction from our data was that if it were possible to block neuroblast PCD, many more neuroblasts should then be present. This would appear as both an enlarged VZ and an increase in the total size of the brain. Several laboratories with immunological interests have created mice null for pro–cell death genes (e.g., caspases). Remarkably, the effects of deleting these genes, particularly individual caspases, did not result in

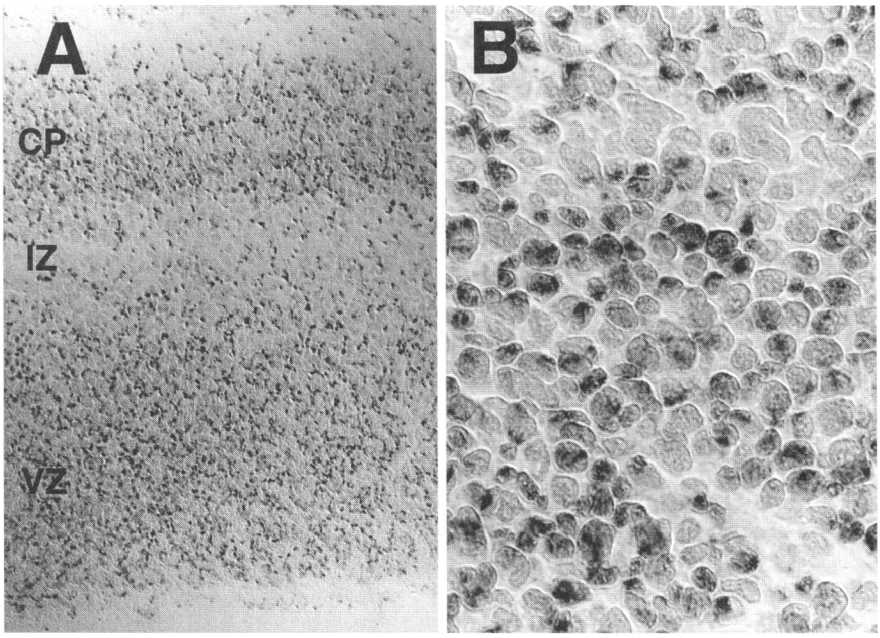

FIG. 7. ISEL$^+$ (*in situ* end-labeling plus) identifies dying cells in the embryonic cerebral cortex, especially apparent in the ventricular zone (VZ). (A) Low-magnification view of the cerebral wall, pial surface to the top. Note the presence of dying cells in the cortical plate (CP), the intermediate zone (IZ), and the VZ. (B) Higher magnification of VZ dying cells.

obvious effects to immunological tissues. However, in dramatic contrast, deletion of caspase 3 (Kuida *et al.*, 1996; Woo *et al.*, 1998), caspase 9 (Hakem *et al.*, 1998; Kuida *et al.*, 1998) and Apaf1 (Cecconi *et al.*, 1998; Yoshida *et al.*, 1998) all resulted in increased neuroblast numbers and enlarged brains at ages that preceded the generation of most postmitotic neurons. In addition, one would expect a loss of ISEL$^+$-labeled cells in caspase-null mice, and indeed, this has been formally demonstrated, at least for caspase 3 (Pompeiano *et al.*, 2000).

It is somewhat surprising that these caspase deletion studies did not produce an analogous increase of cell number and size in immunological tissues known to undergo PCD (e.g., the thymus). Whether this represents caspase-independent PCD or the requirement for deletion of multiple caspases simultaneously is unknown. However, the observation that deletion of a single pro–cell death gene produces a nervous system phenotype but not a related immunological phenotype, despite the operation of extensive PCD in both systems, underscores differences in how PCD is regulated in these two systems. This major difference is tempered by observations using ISEL$^+$ on caspase $3^{-/-}$ embryos, where it is

notable that the block in neuroblast PCD is far from complete, approximating a 30% reduction in dying cells (Pompeiano *et al.*, 2000). Therefore, despite the obvious phenotype, a majority of PCD in this system remains and is not dependent on caspase 3 alone. As in the thymus, whether this majority of neuroblast PCD reflects caspase-independent mechanisms or the operation of multiple caspases remains to be determined. One interesting corollary of the results of caspase deletion is that it demonstrates differential susceptibilities of VZ neuroblasts to alterations in cell death machinery; that is, not all cells are affected. This may be due to the known developmental heterogeneity within the embryonic cortex, or possibly molecular heterogeneity of individual neuroblasts, at least with respect to their employed PCD pathways. These possibilities remain open questions that have relevance to recent, intriguing data on the immunological and nervous system defects observed in mouse null mutations of NHEJ genes.

VI. Nonhomologous End-Joining and DNA Rearrangement

Most molecules and cellular functions common to both the immune and nervous systems are also shared with other cell types. However, a mechanism fundamental to the normal development of B and T lymphocytes—somatic DNA rearrangement, which includes both V(D)J recombination and heavy-chain class switching—has thus far distinguished lymphocytes from all other somatic cells. In the immune system, failed or inappropriate DNA rearrangement results in PCD. V(D)J recombination in lymphocytes is necessary in the assembly of antigen receptor genes encoding Ig's and TCRs. Two events are necessary in order for DNA rearrangement to occur: DNA cleavage, then joining of cleaved free ends to produce the rearrangement. The enzymatic machinery required for the first part of the reaction requires the recombinase proteins RAG1 and RAG2 (Schatz, 1997), which cut DNA at precise, site-specific locations positioned next to gene segments to be recombined. This reaction produces double-stranded breaks in DNA that are blunt and 5′-phosphorylated (Schlissel *et al.*, 1993). The second major step recombines the DNA ends that have characteristic modifications, depending on whether they are coding joins, which form hairpins, or signal joins, which are precise and often produce recircularization of the cleaved byproduct (Schatz, 1997; Schatz *et al.*, 1992). This NHEJ reaction requires several identified proteins: XRCC4 and ligase IV, which form a heterodimer (Critchlow, *et al.*, 1997; Grawunder *et al.*, 1997); Ku proteins (a heterodimer of Ku70 and Ku80); and DNA-dependent protein kinase.

Studies from several laboratories, including our own, have analyzed genes involved with DNA rearrangement in studies of the nervous system or as an adjunct to immunological studies. Genes associated with the cleavage reaction—RAG1 and RAG2—do not produce obvious brain phenotypes, despite clear disruption of V(D)J, in null mutants (Mombaerts *et al.*, 1992; Shinkai *et al.*,

1992). Neural expression of RAG1 (but not RAG2) and indirect effects related to Ig light chain (Chun *et al.*, 1991; Weiner and Chun, 1997a) or potential DNA transposition (Agrawal *et al.*, 1998) leave open possible nonessential functions for RAG1. However, the actual function of RAG1 in the nervous system is not known.

In dramatic contrast, deletion of genes involved with NHEJ, thought to be ubiquitous in expression, produce not only V(D)J disruption but also a remarkable phenotype within the CNS: early death of embryonic neurons (Barnes *et al.*, 1998; Gao *et al.*, 1998; Gu *et al.*, 2000). The effect is obvious even using the same histological techniques that were incapable of accurately identifying PCD in the normal thymus or embryonic brain (noted above). More sensitive PCD detection techniques, such as $ISEL^+$, have not been reported for these mutants. However, the presence of pyknotic cells within or just basal to the VZ indicates that their demise commenced within the VZ (Fig. 8), when the cells were neuroblasts (discussed in Chun and Schatz, 1999a,b). The actual percentage of neuroblasts that may be susceptible to NHEJ mutations is difficult to determine, since the most severe phenotypes—XRCC4 or ligase IV (null genotypes)—are also associated with embryonic lethality that ultimately results in the death of all cells. Because of this, the developmental point at which cell death is assessed in these null mutants will influence the extent of death observed.

What is also clear in these studies is that NHEJ defects do not uniformly affect all developing neurons simultaneously (discussed in Chun and Schatz, 1999a,b) Indeed, based on results from Ku-deficient animals (Gu *et al.*, 2000), it would appear that increased neuroblast loss (milder than XRCC4 or ligase IV, but still present) is compatible with a normal-appearing brain. This indicates that the lost cells were not needed for normal brain function, representing a dispensible subpopulation. In light of the absence of PCD effects in both the thymus and the majority of neuroblasts in caspase 3–deficient mice, the heterogeneity in NHEJ mutant neuroblast PCD is not unexpected. By contrast, T cells deficient for NHEJ genes still show uniform death that is accompanied by clear disruption of the actual NHEJ associated with V(D)J recombination (Frank *et al.*, 1998; Gao *et al.*, 1998; Gu *et al.*, 2000).

This raises the question of whether NHEJ is also disrupted in neuroblasts. There is no direct way to address this question in the absence of a known, endogenous target locus. However, a positive correlation exists between the extent of defective NHEJ and PCD in lymphocytes, and the severity of neuroblast PCD (Gao *et al.*, 1998; Gu *et al.*, 2000), which is consistent with involvement of NHEJ. On the other hand, mechanisms of NHEJ PCD in neuroblasts may not be cell autonomous or may be defective in growth control, as suggested for yeast deficient in $lig4^{-/-}$(Barnes *et al.*, 1998). Recent studies demonstrating that the absence of p53 can rescue neuronal cell death in a NHEJ-null genotype (XRCC4), but not death in T cells (Gao *et al.*, 2000) further complicates

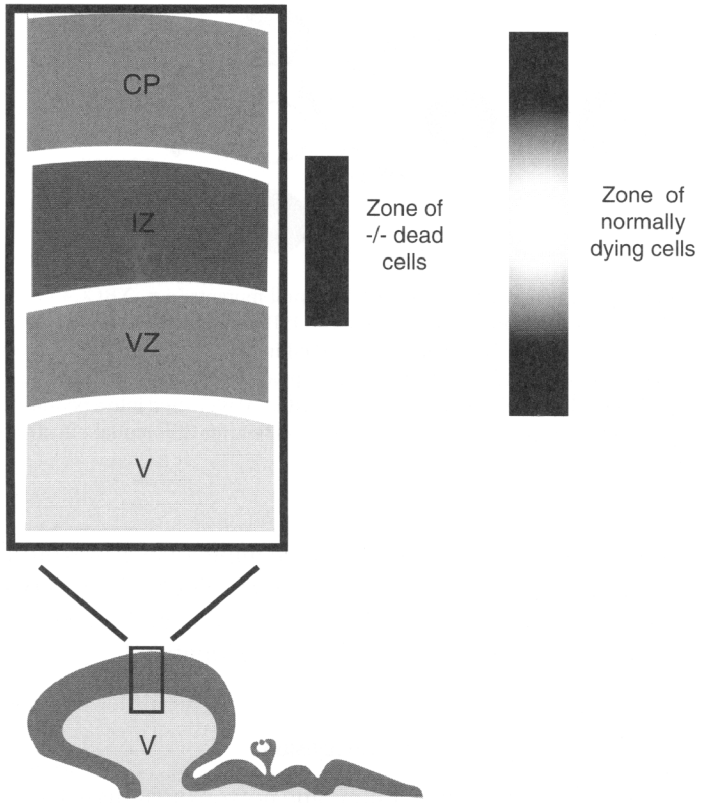

FIG. 8. Distribution of dead cells in NHEJ-null mutant cerebral cortices, as compared to the normal distribution of dying cells identified by ISEL$^+$ (*in situ* end-labeling plus). CP, Cortical plate; IZ, intermediate zone; V, ventricle; VZ, ventricular zone.

simple models of how neuroblasts are affected by the loss of NHEJ components (Fig. 9). T cells clearly have different requirements, compared to neuroblasts, for molecular death signals such as caspases. Moreover, even within a population of neuroblasts, there appear to be different requirements for both caspase death signals and NHEJ components. All these differences represent uncertainties that will no doubt be clarified in coming years.

Initial postulates of nervous system DNA recombination in the 1960s (Dreyer *et al.*, 1967) envisioned changes related to axonal connections during regrowth of axons into the tectum (midbrain) of goldfish. No locus for DNA rearrangement has yet been identified, but the best data to support such a mechanism to date, are the effects of NHEJ mutants on neuroblast PCD before the outgrowth of axons. Whether an actual DNA rearrangement is taking place is purely hypothetical but

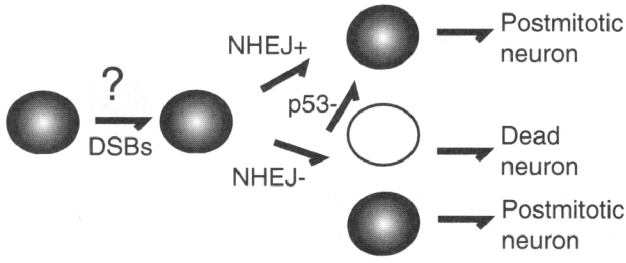

FIG. 9. Summary of neuroblast effects in NHEJ-null mutants (e.g., XRCC4 or ligase IV) with or without p53. The DNA double-stranded breaks (DSBs) are somehow produced during neurogenesis. In the absence of NHEJ components, marked increases in cell death, detectable even by standard histology, are observed. In addition, many apparently unaffected neurons also remain in these mutants. If the NHEJ-null mutant (at least for XRCC4) is crossed into a p53-null background, the neuroblast death phenotype is rescued. The explanation for differential effects on neuroblast subpopulations is not known.

would likely be distinct compared to V(D)J recombination, based on all available evidence. It must be noted, however, that the complexity of the nervous system combined with cellular and molecular differences noted here leaves open the possibility that DNA rearrangements may yet be identified.

VII. Conclusion

Comparison of the developing immune and nervous systems reveals significant similarities but also significant differences. Many analogous functions exist, as do shared molecules. The closest similarities appear to be between the ongoing cellular interactions of double-positive T cells and those of embryonic neuroblasts during the generation of postmitotic neurons. The operation of PCD in both systems demonstrates varying susceptibilities to the functions of caspases, NHEJ molecules, and p53 as well as efforts to understand the mechanisms behind both similarities and differences provide a fertile area for current research. The issue of DNA rearrangement in neurons remains a viable possibility, especially considering the involvement of NHEJ in subpopulations of developing neuroblasts and neurons. However, if this occurs, it will be distinct from V(D)J recombination, and the only suitable proof will be the identification of a rearranged genomic locus within a normal neuron.

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