

Rearranging Views on Neurogenesis: Neuronal Death in the Absence of DNA End-Joining Proteins

Minireview

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Embryonic neurogenesis in mammals occurs over a discrete developmental period from which many different neuronal types arise. In the central nervous system (CNS), newly postmitotic neurons are "born" in an anatomically defined proliferative region overlying the ventricles, as epitomized in the cerebral cortex by the ventricular zone (VZ; Figure 1A). After birth, a newly postmitotic neuron migrates superficially through the intermediate zone (IZ) to locate in the future cortical gray matter, the cortical plate (CP). Three general processes occur among embryonic blasts within the VZ: cell proliferation, differentiation, and death. The general molecular mechanisms thus far described for these cellular fates have not been vastly different from those occurring in other proliferating epithelia, including important roles for growth factors, transcription factors, and cell death molecules. This poses the question of how and when the vast extent of neuronal heterogeneity arises from the VZ. Is there a distinguishing mechanism to account for this, and might it involve a genetic generator of diversity that breaks and reconfigures chromosomes, in a manner analogous to the V(D)J recombination reaction used by lymphocytes to generate diverse antigen receptors? A provocative, recent study from the Alt laboratory (Gao et al., 1998) suggests that chromosomal breaks may indeed be generated and repaired at a specific stage of neurogenesis, around the point where neurons become postmitotic in the VZ but before reaching the CP. The evidence: two DNA double strand break repair (DSBR) proteins that are critical for V(D)J recombination are also involved in the survival of neurons arising from the VZ. Here, we discuss the implications of this intriguing finding in the context of what is known about recombination in lymphocytes, the DSBR machinery, and relevant aspects of embryonic brain development.

There are two general mechanisms by which DNA double strand breaks (DSBs) are generated: specific DNA cleavage reactions, such as occur during V(D)J recombination; and DNA damage, such as that caused by ionizing radiation. Once breaks are generated, there are two general strategies for repairing them: homologous recombination, which typically makes use of genetic information contributed by a homologous chromosome or chromatid; and nonhomologous end joining

(NHEJ), in which the broken DNA ends are rejoined, often imprecisely. Both mechanisms appear to operate in all eukaryotes, with NHEJ most important in the G0, G1, and early S phases of the cell cycle, and homologous recombination operating predominantly in late S and G2 (Hendrickson, 1997). Efficient repair of DSBs is of paramount importance since even a single chromosomal break can be a lethal event for a cell.

In the December 23 issue of *Cell*, Gao et al. (1998) present results from the most recent of several related studies that have used gene "knockout" strategies in mice to produce null mutations in genes involved in NHEJ. The principal gene analyzed in the paper, *XRCC4*, was first identified based on its ability to complement a mutant cell line defective in V(D)J recombination and DNA DSB repair (Li et al., 1995). It was, therefore, not surprising that *XRCC4*-deficient mice were defective in V(D)J recombination (with a corresponding block in lymphocyte development) and hypersensitive to ionizing radiation (Gao et al., 1998). However, unlike the deletion of

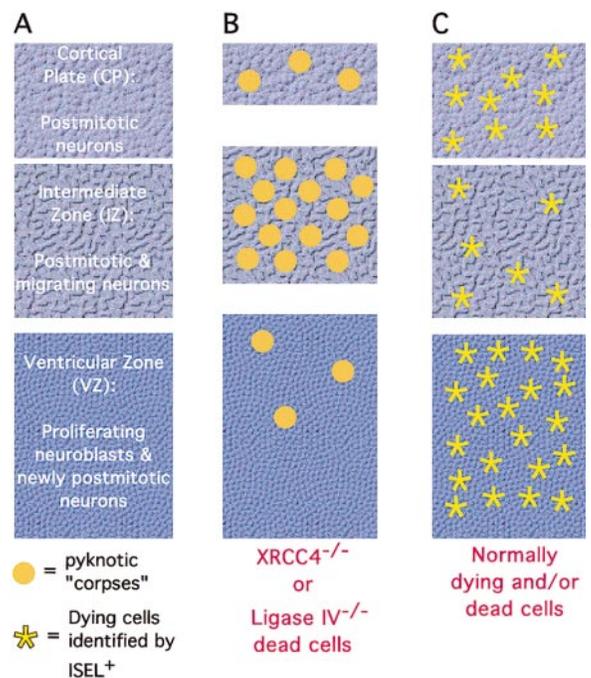


Figure 1. Cell Death in the Embryonic Cerebral Cortex

(A) The embryonic neurogenic region of the cerebral cortex, the ventricular zone (VZ) surrounds the lateral ventricles and gives rise to cells of the cerebral cortex, with the vast majority of neurons arising through marked cell proliferation before birth. Within the VZ, neuroblasts give rise to postmitotic neurons that migrate superficially through the intermediate zone (IZ) to locate in the cortical plate (CP), the future gray matter that will contain adult neurons.

(B) Schematic representation of pyknotic cells observed in *XRCC4*^{-/-} and *LigIV*^{-/-} brains at comparable ages (adapted from Gao et al., 1998).

(C) Schematic representation of dying cells identified by the more sensitive technique, in situ end labeling plus (ISEL⁺), that occur normally in the embryonic cortex (Blaschke et al., 1996). Note the presence of apoptosis in both postmitotic and proliferative regions of the cerebral wall.

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related genes, the loss of *XRCC4* was associated with embryonic lethality. The cause of death was not immunological, but, surprisingly, was due to a rather specific and pronounced defect throughout the embryonic nervous system: many early, postmitotic neurons, identified by anatomical location and correlated β 3-tubulin immunostaining, were dead (Figure 1B). The same nervous system phenotype was also observed in animals null for DNA ligase IV (Frank et al., 1998; Gao et al., 1998), the dimerization partner of *XRCC4*. Based on the functional properties of these proteins, and the fact that independently derived mutations of each gene produced the same phenotype in both the immune system (as expected) and the nervous system, it seems likely that the early neuronal death in both mutants is due to a defect in NHEJ, and hence the presence of unrepaired DSBs, in these neurons.

One well-characterized source of DNA DSBs is V(D)J recombination, which assembles immunoglobulin and T cell receptor genes from component gene segments. In the first phase of this reaction, the proteins encoded by the recombination activating genes, *RAG1* and *RAG2*, cleave a chromosome in two locations, immediately adjacent to the two gene segments to be recombined (Figure 2A). In the second phase of the reaction, the four broken ends are rejoined in a new configuration, resulting in the assembly of a potentially functional antigen receptor gene. The second, end-joining phase of V(D)J recombination is critically dependent on ubiquitous NHEJ factors, including Ku protein, the DNA dependent protein kinase (DNAPK), and the *XRCC4*/ligase IV heterodimer, and hence is thought to closely resemble NHEJ in mechanism (Figure 2B). While the process of NHEJ is poorly understood, the biochemical and genetic evidence strongly suggest that DNA ligase IV performs the final, ligation step of the reaction (Frank et al., 1998; Grawunder et al., 1998), apparently as a tight heterodimer with *XRCC4* (Critchlow et al., 1997; Grawunder et al., 1997). *XRCC4* is able to stimulate the activity of the ligase (Grawunder et al., 1997), but its other functions, if any, have not been determined.

V(D)J recombination occurs exclusively in lymphocytes, most notably in B and T cell precursors in the bone marrow and thymus, respectively. The stochastic nature of the recombination process results in a highly diverse array of antigen receptors, which are then the target of multiple selective processes that eliminate those with useless or potentially harmful (self-reactive) specificities. Selection is best understood in the thymus, where the vast majority of developing T cells die, either through negative selection or because of a failure to receive a survival signal (death by neglect). The survival or death of thymocytes, therefore, can be directly traced to the fine specificity of the T cell receptors they express on their surface, and hence to the V(D)J recombination events that gave rise to the receptor genes.

Prior Conceptions of Nervous System DNA Rearrangements

The theory that the nervous system might utilize some form of somatic DNA rearrangement mechanism (Dreyer et al., 1967) precedes by nearly a decade its actual demonstration for immunoglobulin genes (Hozumi and Tonegawa, 1976). Dreyer, Gray, and Hood (1967) envisaged a "copy-splice" DNA rearrangement mechanism

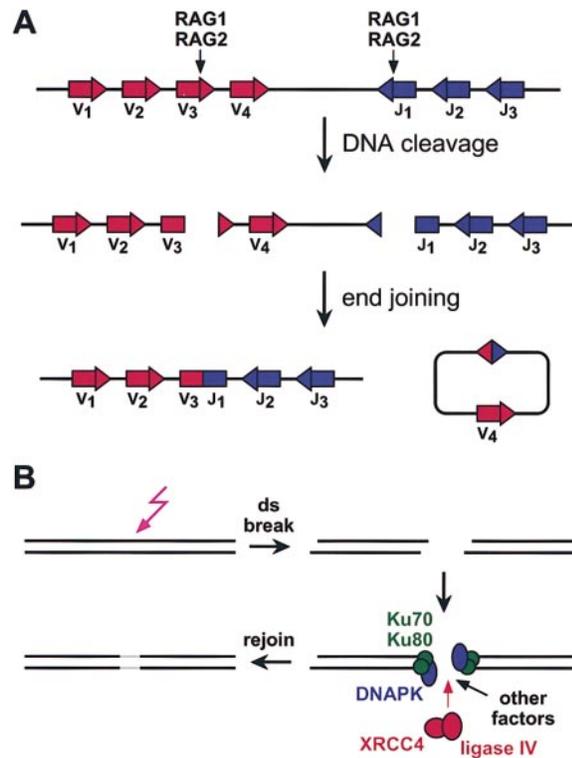


Figure 2. V(D)J Recombination and Nonhomologous End Joining (NHEJ)

(A) V(D)J recombination of a hypothetical antigen receptor locus containing four variable (V) and three joining (J) gene segments. *RAG1* and *RAG2* select at random one of each type of gene segment (here, V3 and J1) and cleave between the gene segments (rectangles) and flanking recombination signals (triangles). The NHEJ machinery then joins the two coding segments to create a complete antigen receptor gene, and fuses the two recombination signals, deleting a portion of the chromosome as a circle. In some instances, V(D)J recombination results in chromosomal inversion rather than deletion.

(B) Repair of a DNA double strand break by NHEJ. The DNA ends generated during V(D)J recombination or by ionizing radiation (jagged arrow) provide high-affinity binding sites for Ku (a heterodimer of Ku70 and Ku80 subunits) and subsequently DNAPK. Ku and DNAPK may help tether the two ends together and signal the presence of DNA damage (perhaps via the serine/threonine protein kinase activity of DNAPK). Other factors are also recruited to the site of the lesion, which may assist in processing and aligning the two ends. Finally, the *XRCC4*/ligase IV heterodimer ligates the two ends together.

to produce "surface displays" of molecules that could allow the ordered regeneration of postmitotic retinal ganglion cell neurons as they reinnervated the tectum in goldfish. More recently, the discovery of a highly diverse family of odorant receptors has stimulated speculation that DNA rearrangement may be involved in determining which receptor is expressed in each olfactory sensory neuron (Buck and Axel, 1991). It remains the case, however, that unlike immunoglobulins and T cell receptors of the immune system, the nervous system does not have an obvious target molecule that might be expected to be diversified by DNA rearrangements. Add to this the fact that postmitotic neurons cannot be clonally expanded like a cell line, along with the lack of markers

known to be linked to a rearrangement event, and identification of a neuronal surface display molecule becomes difficult indeed, assuming that it even exists.

With the identification of *RAG1* and *RAG2*, it became possible to ask whether genes involved in somatic recombination in lymphocytes were expressed in the nervous system, as an indirect assessment of recombination potential. Indeed, *RAG1* is expressed, albeit at very low levels, particularly in parts of the brain containing proliferating blasts and early postmitotic neurons: the embryonic CNS and early postnatal cerebellum and hippocampus (Chun et al., 1991). *RAG2* is not appreciably expressed in the CNS, indicating that if *RAG1* is participating in DNA recognition and cleavage, it must be of a distinctly different form than that which occurs in the immune system. Genetic deletion of *RAG1* in mice does not produce an obvious CNS phenotype (Mombaerts et al., 1992). However, the fact that *RAG1* is expressed in neurogenic regions suggests that it might have nonessential functions in early events associated with neuroproliferation and differentiation to influence the production of postmitotic neurons. Curiously, *XRCC4* or ligase IV deficiency affects embryonic neuronal cells in the same general anatomical locations as one finds *RAG1* transcripts (Gao et al., 1998). The significance of this correlation remains to be determined. Postnatal neurogenic compartments cannot be similarly compared, since *XRCC4* and *LigIV* mutants die before birth. It would not be surprising, however, to find a similar loss of newly postmitotic neurons in postnatal neurogenic populations, a possibility that could be addressed with conditional mutants.

Extensive Apoptosis among Proliferating and Early Postmitotic Neurons

One conspicuous similarity between lymphogenesis and neurogenesis is the involvement of extensive apoptosis. In the embryonic brain, apoptosis affects differentiating, postmitotic neurons (Hamburger, 1975), and, to an even greater extent, neuroblasts undergoing cell cycle progression or early postmitotic differentiation preceding axon outgrowth (Blaschke et al., 1996, 1998; Kuida et al., 1996, 1998). Adult neurons thus survive at least two distinct phases of embryonic apoptosis, one associated with their neuroproliferation and early, postmitotic "birth" in the VZ, and the other affecting them as mature neurons. This is illustrated by the location of dying cells in the embryonic cerebral wall, where they are distributed in neuroproliferative and postmitotic regions (Figure 1C; Blaschke et al., 1996, 1998). These two phases of apoptosis can be further distinguished not only by the anatomical location of dying cells, but by cellular features: neuroproliferative apoptosis does not involve synaptic competition, nor does it involve classical trophic competition or activity-dependent competition of the form where neurons utilize axons projecting to distant targets. Independent support for this form of apoptosis comes from the phenotype of caspase-null mice, which show a massive increase in neural cell number at early embryonic stages (Kuida et al., 1996, 1998). During neurogenesis, 50% or more of cells are dying, with the substantial loss compensated for by the exponential increase in cells being produced over this same period (Voyvodic, 1996). The early neuronal death observed by

Gao et al. (Figure 1B) parallels normal, neuroproliferative apoptosis in three ways: it occurs throughout the nervous system, is nearly absent before the start of neurogenesis, and commences with the onset of neuronal differentiation when blasts become postmitotic.

It is important to note that Gao et al. examined apoptosis in the *XRCC4*- and ligase IV-deficient mice using histological stains or TUNEL, methods that visualize pyknotic nuclei. Pyknotic nuclei may be thought of as cell "corpses," representing an end stage of the apoptotic process (Voyvodic, 1996). A corollary of this is that the corpses started dying a good bit earlier. The distribution of the *XRCC4*/ligase IV cellular corpses is telling: they are predominantly observed *below* the CP in the IZ and appear to extend into the VZ itself (e.g., Figure 4 in Gao et al., 1998). This indicates that the majority of affected cells began dying before being found as corpses below the CP, which, based on the normal distribution of dying cells (cf. Figures 1B and 1C), suggests that at least some of this death could have been initiated in the VZ. It is also apparent that most of the affected neurons never reached their normal destination, the CP, as seen by the reduction in the width of the CP itself. The determination of where death was initiated is important, since this would help to identify the location and developmental stage of the cell at the time of a putative DNA cleavage event that would normally be repaired by NHEJ. It would also provide information about the proliferative capacity of the cells that gave rise to the corpses. One factor that may complicate these analyses is that not only do all mutant embryos die before birth, but, even at earlier embryonic ages, the Mendelian ratio is skewed away from homozygous null animals (Table 1 in Gao et al., 1998). Quantitation of dying or dead cells could therefore be affected by the state of health of the embryo.

Despite these uncertainties, two aspects of the neuronal loss in *XRCC4*- or ligase IV-deficient mice are clear. First, death occurs on a large scale before a young neuron can reach its final postmitotic position, and in some instances before the neuron leaves the VZ, demonstrating the initiation of apoptosis well before a neuron achieves maturity. Second, not all neurons die at the same time, and, at least based on histological stains, the survivors appear healthy (Figure 6I, hindbrain, in Gao et al., 1998). This latter aspect suggests either that not all neurons require these NHEJ proteins or that some cells can make use of a compensatory mechanism. Perhaps the first-born neurons such as those of the cortical subplate (Shatz et al., 1988) do not have a requisite reliance on NHEJ proteins compared to later-generated neurons, a possibility supported by the comparative lack of pyknotic profiles at the earliest ages of cortical development examined (Figure 6b, embryonic day 11.5, in Gao et al., 1998). Whatever the reason, this heterogeneity of susceptibility deserves further study.

Loose Ends

There are a number of parallels between lymphocyte and neuronal development, notably the reliance on extensive cellular proliferation and death, and now the requirement for *XRCC4* and DNA ligase IV. Do they extend so far as to include the generation of specific chromosomal breaks, and perhaps even a generator of diversity? Possibly, but as noted by Gao et al. (1998), there are other

explanations available for neuronal death in the *XRCC4*- and *ligase IV*-deficient mice. One formal possibility, for which there is as yet no evidence, is that *XRCC4* and *ligase IV* have a role in a previously unsuspected process, distinct from NHEJ. This would be rendered unlikely if deficiencies in other NHEJ factors led to an equivalent neuronal phenotype. Interestingly, *Ku*- or *DNAPK*-null mice do not exhibit embryonic lethality, and it will be of considerable interest to examine these mice during embryogenesis and postnatally for evidence of increased neuronal cell death. Another possibility is that the observed cell death is not cell autonomous but instead occurs because the NHEJ deficiency leads to a defect in some other cell type (for example, preventing production of a required growth factor), which then manifests itself as early neuronal cell death. This would be a surprising departure from what is observed in the immune system. Yet another possibility is that neurons pass through a stage of development at which they are particularly sensitive to DSBs. Gao et al. point out that there is evidence that neurons are most sensitive to ionizing radiation immediately after they are born (Bayer and Altman, 1991). Such a window of DSB hypersensitivity might indicate that neurons die in the *XRCC4* and *LigIV* mutants because a small number of DSBs, created by normal, nonspecific processes, go unrepaired. Alternatively, it might indicate that these neurons already contain an unusually high load of DSBs (e.g., generated by a site-specific recombination process) and hence are least able to cope with additional damage (Blaschke et al., 1996).

The striking results of Gao et al. (1998) force one to confront more seriously than ever before the possibility that neuronal genomes are cleaved and rejoined as part of normal neurogenesis. We speculate that this could offer an explanation for the extensive neuronal apoptosis that occurs in the normal developing brain, just as it does in the immune system: it is a reflection of a selective process that sorts out those cells making advantageous recombination products from those that do not. Serious thinking about the physiological role of such a process must await the identification of a putative rearranging locus, but it would not be surprising if the locus encoded a cell surface molecule with the ability to signal survival or death.

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