The Role of Cell Death in Regulating the Size and Shape of the Mammalian Forebrain

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The size of the cerebral cortex is determined by the rate of production of neurons and glial cells in the proliferative ventricular and subventricular zones. Recent studies from targeted mutations of different death-effector gene families indicate that programmed cell death (PCD) plays an important role in cell production and early morphogenesis of the mammalian forebrain before the formation of neuronal connections. For example, disruption of the c-*Jun* N-kinase signaling pathway by double-targeted mutation of both *Jnk1* and *Jnk2* results in increased PCD in the forebrain leading to precocious degeneration of cerebral precursors. In contrast, disturbance of the caspase cascade by targeted disruption of either *casp-9* or *casp-3* leads to decreased PCD causing expansion and exencephaly of the forebrain as well as supernumerary neurons in the cerebral cortex. The supernumerary neurons in these knockout mice align radially and form an expanded cortical plate which begins to form cerebral convolutions. Thus, the precise coordination of different apoptotic signaling pathways during early stages of neurogenesis is crucial for regulation of the proper cortical size and shape.

Introduction

The mammalian forebrain, while remaining remarkably consistent in basic structural design, varies greatly in the number of neurons and surface area of the cerebral cortex between different species. The increase in surface area of the cerebral cortex of certain species, e.g. non-human primates, is accompanied by the addition and elaboration of cytoarchitectonic areas that have intricate connections (Felleman and Van Essen, 1991). However, before this parcellation occurs the proper number of cortical neurons have to be produced (Rakic, 1995). In all mammals, the cerebral cortex is generated from the proliferative ventricular zone (VZ) and subventricular zone (SVZ) which line the lateral ventricles (His, 1889; Smart, 1972; Sidman and Rakic, 1982). Neuronal cells born in the VZ and SVZ then migrate outward to form the forebrain cortical plate (Rakic, 1972; Sidman and Rakic, 1973). Much of the interspecies diversity in forebrain size arises from differences in several cell cycle parameters which effect the rapidly dividing progenitor population during early development of the brain (Takahashi *et al.*, 1997). These parameters include cell cycle duration, the length of the pre-neurogenetic period of development during which progenitor number increases exponentially, and the ratio of symmetric and asymmetric cell divisions during neurogenesis (Caviness *et al.*, 1995; Rakic, 1995). In addition to these proliferative parameters, programmed cell death (PCD) may also be used to specify the size of the central nervous system (CNS) during development (Strom and Williams, 1998). In this paper, we will discuss how recent evidence indicates that PCD, both before and during neurogenesis, plays a critical role in determining the appropriate species-specific size and shape of the forebrain.

PCD may have several functions during normal development of the mammalian brain. First, spatially regulated cell death before neurogenesis has been postulated to contribute to the complex three-dimensional brain morphogenesis by eliminating intervening cell populations and thereby allowing the associated flexures to oppose or separate populations of cells (Glucksmann, 1951; Kallen, 1955). In addition, even small amounts of morphogenetic cell death during the early exponential growth phase of the progenitor population in the VZ can drastically effect the eventual size of the neuronal population in the adult cerebrum (Rakic, 1988). Second, histogenetic cell death, which occurs during and after neurogenesis, may be involved in matching the size of neuronal populations to that of their targets during the formation and maintenance of synapses (Burek and Oppenheim, 1996; Sherrard and Bower, 1998). Lastly, in addition to the roles of apoptosis during normal development, dysregulated or pathogenetic cell death may underlie the etiology of congenital diseases. For example, abnormally high rates of apoptosis occur in Down syndrome both during and after forebrain neurogenesis (Wisniewski and Kida, 1994; Busciglio and Yankner, 1995). Thus, delineating the signaling pathways and temporal controls of apoptosis during forebrain development can provide insights into the mechanisms of both normal growth and the pathogenesis of congenital brain malformations.

The signaling pathways that control these different types of developmental apoptosis can be separated into extracellular and intracellular components. Although little is known concerning the external molecules which initiate these apoptotic events, they most probably act via contact-mediated or trophic mechanisms mediated by neighboring cells or target cells. Conversely, several of the intracellular regulatory pathways have been elucidated recently, including the c-*Jun* N-terminal kinase (Jnk) and caspase-mediated apoptotic mechanisms. Here we describe how targeted mutations of these intracellular pathways has revealed the importance of developmental PCD in specifying proper forebrain development.

The Jnk Pathway Regulates Region-specific Cell Death in the Brain

The *Jnk* family consists of at least ten isoforms generated by alternative splicing of three genes, *Jnk1*, *Jnk2* and *Jnk3* (Gupta *et al.*, 1996). *Jnk1* and *Jnk2* are widely expressed in various tissues, whereas *Jnk3* expression is mainly restricted to the nervous system (Ip and Davis, 1998). Jnk protein kinases target several apoptosis-related proteins, including c-Jun, p53 and Bcl-2, and can activate the caspase response (see below). These proteins are therefore likely involved in apoptotic regulation *in vivo*. Most importantly, all three *Jnk* genes are expressed early in development, suggesting a role in the regulation of the early developmental apoptosis. We tested this hypothesis by generating compound mutants using previously established single-knockout animals for *Jnk1* (Dong *et al.*, 1998), *Jnk2* (Yang *et al.*, 1998) and *Jnk3* (Yang *et al.*, 1997) and assessing forebrain

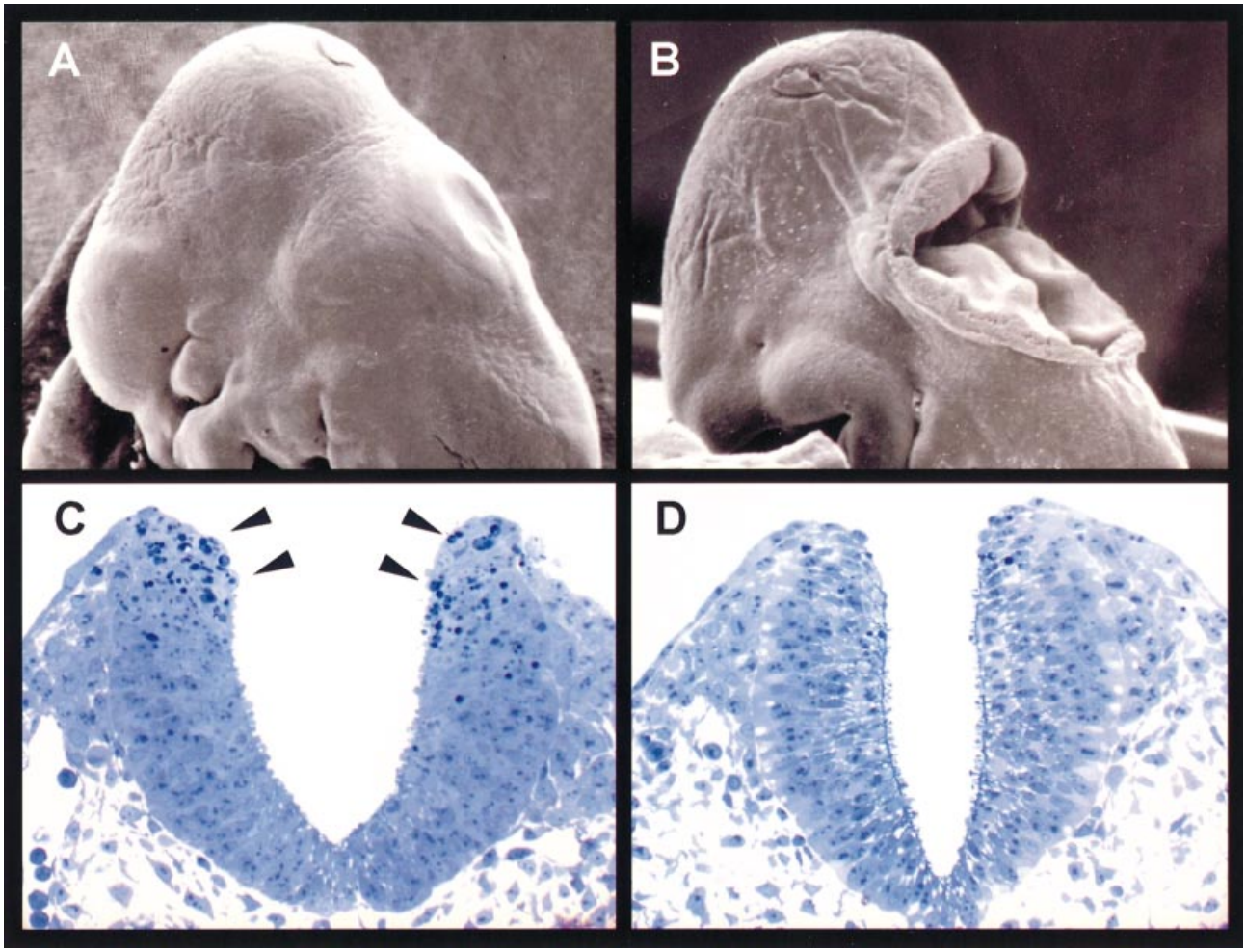

Figure 1. The Jnk pathway mediates region-specific death in the hindbrain. At E11.5, the hindbrain neural tube is closed in the wild-type embryo (*A*) while the neural tube remains open in *Jnk1*$^{-/-}$/*Jnk2*$^{-/-}$. (*C*) During neural tube closure at E9.0, pyknotic profiles of apoptotic cells (arrowheads) are clustered at the lateral edges of the neuroepithelial wall in wild-type controls. (*D*) In contrast, few pyknotic cells are spread throughout the dorsal–ventral extent of the neural tube and not clustered in the compound *Jnk*-deficient animals. [Modified from Kuan *et al.* (Kuan *et al.*, 1999).]

development before neurogenesis in *Jnk1/Jnk2*, *Jnk1/Jnk3* and *Jnk2/Jnk3* double knockouts (Kuan *et al.*, 1999). Of the three *Jnk* compound mutants generated, only the double *Jnk1/2* mutants exhibited visible brain abnormalities. Thus, even though *Jnk3* is the only *Jnk* member expressed solely within the CNS, it is *Jnk1* and *Jnk2* that are critical for CNS development (Kuan *et al.*, 1999). We analyzed several brain regions in *Jnk1*$^{-/-}$/*Jnk2*$^{-/-}$ animals throughout development, including the hindbrain and forebrain, to determine where and when these genes may contribute to brain growth.

Beginning at embryonic day (E) 10, *Jnk1/Jnk2*-deficient animals exhibited defective neural tube closure in the hindbrain (Fig. 1*A*,*B*). Prior to neural tube closure, there is clustering of apoptotic cells in the lateral edge of the hindbrain neural tube in normal individuals. Through analysis of pyknotic cells in serial semithin sections, we found that this clustering is absent in *Jnk1/2* double knockouts at E9.0 (Fig. 1*C*,*D*). Therefore, as postulated over 40 years ago (Glucksmann, 1951; Kallen, 1955), the correlation between the highly restricted cell death induced by the Jnk signaling pathway and neural tube closure indicates that this regionally specific PCD is crucial for proper cephalic neurulation.

In contrast, a 10-fold increase in PCD was observed in the E10.5–E11.5 *Jnk1*$^{-/-}$/*Jnk2*$^{-/-}$ forebrain visualized both by pyknotic figures and by the terminal dUTP nick end-labeling (TUNEL) technique which measures DNA fragmentation (Fig. 2*A*,*B*,*D*,*E*). As opposed to the localized PCD in the hindbrain, this PCD is widespread with cells dying throughout the entire forebrain neuroepithelial wall causing nearly complete degeneration of this structure by E11.5. Moreover, the increase in forebrain PCD is correlated with Casp-3 activation as measured by staining for the CM-1 antibody (Fig. 2*E*,*F*), which recognizes the 17 kDa cleaved subunit of Casp-3 but not the inactive proenzyme (Kuida *et al.*, 1998; Srinivasan *et al.*, 1998). Interestingly, no increase in PCD was detected in either the midbrain or hindbrain at these developmental ages, suggesting that *Jnk1* and *Jnk2* normally function to preferentially protect forebrain progenitors (Kuan *et al.*, 1999). Thus, it appears that the Jnk pathway plays a dual role during early brain development: promoting the spatial targeting of caspase-mediated apoptotic cell death in the hindbrain while serving a general anti-apoptotic function during forebrain development. It is interesting to note that these two genes have a specific protective effect in the forebrain, the most expanded part of the mammalian CNS where the founder cells of the cerebral cortex are generated. As described in the next section, early events which control the

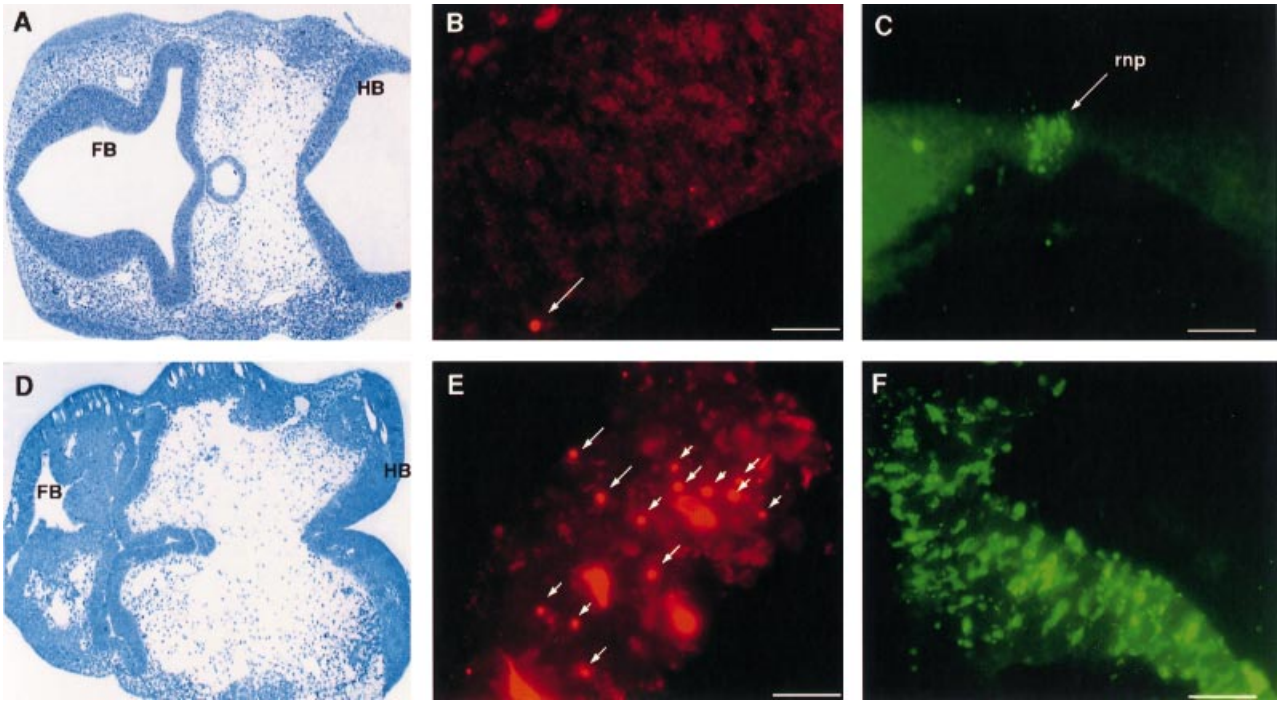

Figure 2. Compound deletion of *Jnk1* and *Jnk2* results in widespread apoptosis in the forebrain neuroepithelium via caspase activation. Few apoptotic cells were found throughout the forebrain (FB) neuroepithelium in wildtype controls at E11.5 (*A*), although all of the sporadic examples were positive for staining using TUNEL (*B*) and CM-1 (*C*), an antibody which recognizes the active form of *Casp-3*. In contrast, complete degeneration of the forebrain was evident in $Jnk1^{-/-}/Jnk2^{-/-}$ embryos (D). This forebrain abnormality is caused by a selective increase in forebrain progenitor apoptosis as measured by TUNEL (*E*) and CM-1 staining (*F*). HB, hindbrain; rnp, rostral neuropore. [Modified from Kuan *et al.* (Kuan *et al.*, 1999).]

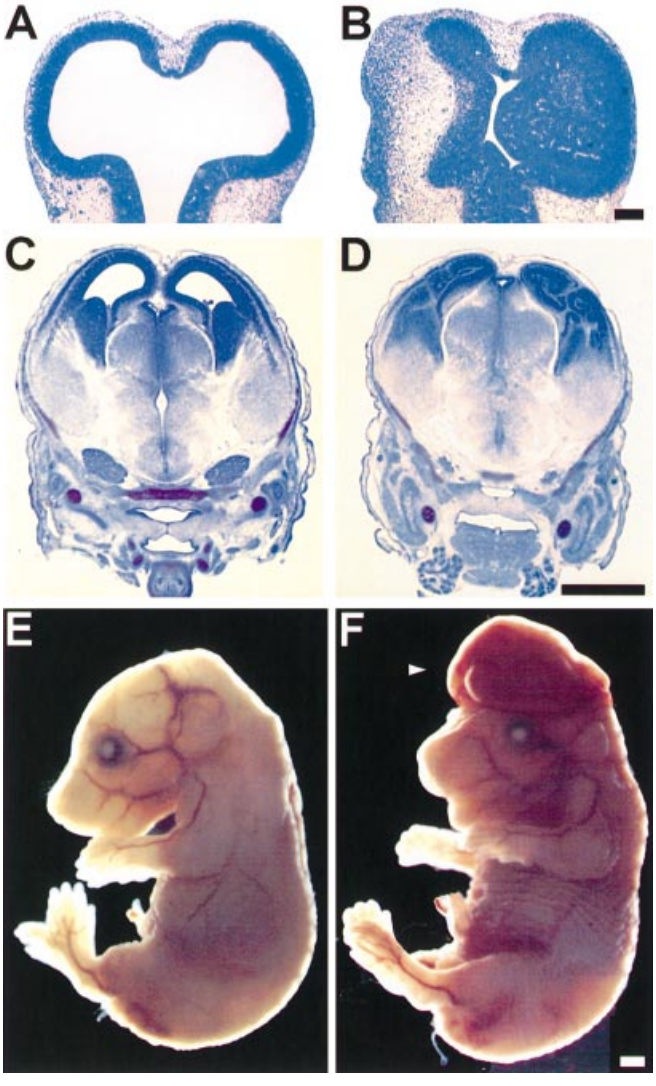

Figure 3. *Casp-9* deletion causes reduced apoptosis and leads to expansion in the number of forebrain progenitors, heterotopic development and exencephaly. (*A*,*C*,*E*) Examples of wildtype individuals at E10.5, E13.5 and E16.5 respectively. At E10.5, histological sections reveal a large overproduction of forebrain progenitors in *Casp-9*-deficient embryos (darkly stained cells in *B*). This expansion of the progenitor pool leads to interruptions and invaginations of the neocortical wall at E13.5 (*D*), and exencephaly by E16.5 (arrowhead in *F*). [Modified from Kuida *et al.* (Kuida *et al.*, 1998).]

number of these founder cells can have a profound effect on the size of the cerebral cortex.

Caspases Control Forebrain Size by Regulating Early Progenitor Cell Death

As outlined in the Introduction, species-specific differences in cortical size depend on a variety of interrelated factors. Here we emphasize only the role of PCD caused by the activation of the caspase family of genes. The caspase cascade, generally thought of as the execution phase of apoptosis (Salvesen and Dixit, 1997; Hengartner, 1998), is initiated by various apoptotic stimuli resulting in cytochrome-c release from mitochondria. Soluble cytochrome-c then activates the cascade whereby different caspase family members are sequentially cleaved and activated (Li *et al.*, 1997; Reed, 1997). One of the first caspases activated is Caspase-9 (Casp-9), and Casp-9 then cleaves and activates Caspase-3 (Casp-3), the penultimate step before the DNA cleavage associated with apoptosis (Li *et al.*, 1997; Kuida *et al.*, 1998; Woo *et al.*, 1998). Like the *Jnk* family members, both *casp-9* and *casp-3*, along with other components of the cascade, are expressed in the brain early during prenatal life and may

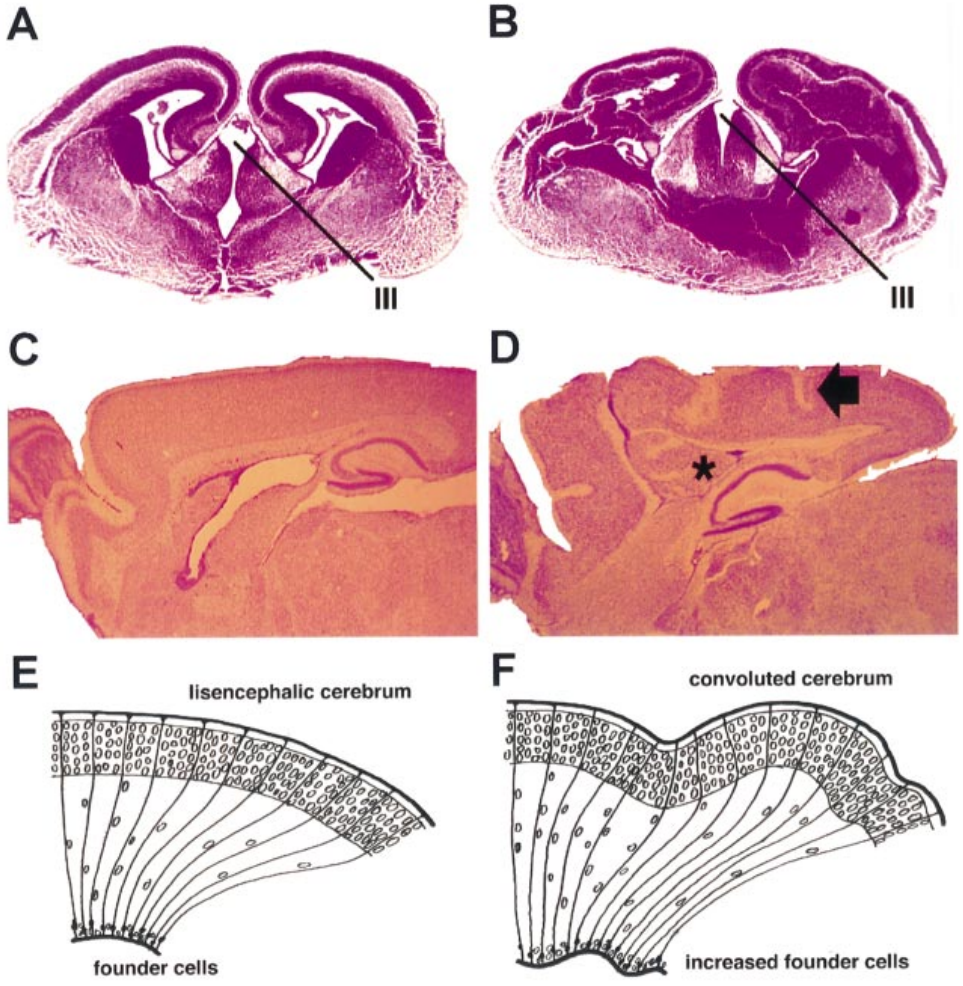

Figure 4. *Casp-3* deletion results in forebrain heterotopias and increased forebrain surface area. (*A*,*B*) Nissl-stained coronal brain sections at E17. The third ventricle (III) is labeled for orientation. Compared to the wildtype littermate (*A*), the section from the *casp-3*$^{-/-}$ individual illustrates the thicker cerebral wall and enlarged ventricular epithelium in these animals. (*C*,*D*) Sagittal Nissl-stained brain sections from wildtype (*C*) and *Casp-3*-deficient individuals at postnatal day 16 illustrate heterotopic cell masses (asterisk) and sulci-like indentations (arrow) of the cortical wall causing an increase in surface area of the mutant cortex. By preventing selected apoptotic deaths in the early forebrain progenitor lineage, caspase deletion causes an increase in forebrain founder cells. Instead of a smooth cerebrum (*E*), this increase in forebrain progenitors leads to a larger expansion of the cerebral wall (*F*) and a convoluted cerebrum of larger surface area. [Modified from Kuida *et al.* (Kuida *et al.*, 1996).]

likewise mediate early apoptotic deaths during forebrain development (Kuida *et al.*, 1996, 1998).

If the caspase cascade is solely a general effector mechanism for apoptotic cell death, it is possible that the specific regional and temporal activation of this pathway during development is controlled by upstream signaling pathways. An example of such regulation is provided by the Jnk pathway above. However, multiple signaling mechanisms may use the caspase cascade to shape the forebrain in still other regions and at other ages. To test this possibility, we analyzed forebrain growth in mice lacking Casp-3 and Casp-9.

Both of the targeted caspase mutations prevented cell death in many regions of the developing brain. In areas where apoptotic cells are typically found during development, including the dorsomedial wall of the diencephalon, the optic stalk and the lamina terminalis, a reduction of pyknotic figures was seen in *casp-3*$^{-/-}$ and *casp-9*$^{-/-}$ embryos (Kuida *et al.*, 1996, 1998). In addition, the few TUNEL^{+} cells found in the neocortical wall during neurogenesis are also absent when the caspase cascade is disrupted (Kuida *et al.*, 1998).

casp-3$^{-/-}$ and *casp-9*$^{-/-}$ mice first exhibit defective closure of the neural tube at E10.5 similar to that found in the compound *Jnk*-deficient mice. This is most likely due to the mutation-induced absence of the previously mentioned cluster of apoptotic cells in the hindbrain neural tube. However, in contrast to *Jnk1*$^{-/-}$/*Jnk2*$^{-/-}$ individuals, as early as E10.5 there is prominent hyperplasia of the forebrain progenitor population in caspase-deficient animals (Fig. 3*A*,*B*). This enlargement of the forebrain progenitor population before the onset of neurogenesis eventually leads to heterotopic neuronal populations and invaginations of the neocortical wall (Figs 3*C*,*D*, 4*A*,*B*) as well as exencephaly of the forebrain during neurogenesis (Fig. 3*E*,*F*).

In addition to the prenatal effects of caspase mutations on the forebrain, postnatal neocortical development in *casp-3*$^{-/-}$ individuals further suggests that early control of apoptosis may have far-reaching implications for adult brain size and shape. At postnatal day 16, the *casp-3*$^{-/-}$ brain exhibits multiple neuronal heterotopias as well as primitive sulci due to an increase in forebrain surface area (Fig. 4*C*,*D*). Thus, the initial abnormality of the larger forebrain progenitor population in caspase-deficient

animals leads to significant expansion of all constituents of neocortical wall, indicating that the caspase cascade is utilized before neurogenesis to control progenitor number and thereby regulate the eventual size of the forebrain.

Importance of Apoptosis for Function and Cortical Evolution

The dramatic increase in cortical size during mammalian evolution is accomplished by expansion of its surface and the formation of convolutions. The genetic and molecular mechanisms which lead to this expansion remain elusive. Nevertheless, apart from the growing number of examples of how genes may regulate cortical size by modulating proliferation, migration and differentiation (Haydar *et al.*, 1996; Gleeson *et al.*, 1998; Lee *et al.*, 1998; Dou *et al.*, 1999; Vaccarino *et al.*, 1999), the results presented here suggest that PCD may also play an important role in forebrain growth. The compartmentalized effects of Jnk and caspase mutations identifies PCD as a mechanism for modulating the production of certain progenitor populations while sparing others. Further, the precise spatial regulation of apoptosis during brain development indicates that differences between progenitors of different CNS regions as well as between progenitors of the same area can be exploited by upstream pathways to control size and shape. For example, while brain regions such as the medulla oblongata increase linearly with increased body weight, disproportionately large increases in forebrain size are observed between humans and other species (Hofman, 1982).

The increased forebrain size of caspase-deficient embryos, due to the abnormal survival of a specific subgroup of forebrain progenitors before neurogenesis, suggests that PCD may be a primary and efficient mechanism for generating the diverse numbers of forebrain cells evident in different species. For example, because of the exponential growth in the progenitor population before the onset of telencephalic neurogenesis, three extra founder cells can generate 192 extra progenitor cells after six cell divisions (Rakic, 1995). These additional progenitors can then significantly increase the number of neurons produced during neurogenesis. We do not claim that PCD is the only, or even the major, factor leading to the increase in cortical size during evolution. However, we suggest that the protection of a small number of specific founder cells may significantly effect the number of neurons generated during later stages of development. In addition, while larger numbers of generated cells may result in cortical convolutions such as those seen in the mutant mice presented here, the precise species-specific pattern of convolution depends also on areal specification and connectivity between the cortex and cortical or subcortical targets (Richman *et al.*, 1975; Goldman-Rakic and Rakic, 1984; Rakic, 1988; Van Essen, 1997).

It is interesting to note that the increase in founder cells present in caspase-deficient embryos translates to a neocortical plate with larger surface area but with normal thickness. This finding suggests that the glial scaffolding present within the neocortical wall constrains the migration and deployment of these additional postmitotic cells into additional radial units (see Fig. 4*C–F*) as predicted by the radial unit hypothesis (Rakic, 1988) rather than expanding the depth of existing units. However, in addition, many presumably later generated cells accumulate below the cortical plate (Fig. 4*D*) in a manner similar to the 'double cortex' observed in human and rodent congenital malformations (Gleeson *et al.*, 1998; Lee *et al.*, 1998).

In contrast to the significant role of PCD in modulating the number of early progenitors before the onset of neurogenesis, the present results suggest that PCD may not play a major role *during* neurogenesis. The selective forebrain degeneration evident in *Jnk1*$^{-/-}$/*Jnk2*$^{-/-}$ embryos (Fig. 2*A*,*D*) indicates that the Jnk pathway normally has a protective effect on neural progenitors while they are producing young neurons. While it is possible that other upstream pathways may be used to regulate apoptosis during forebrain neurogenesis, the small amount of TUNEL and CM-1 staining in normal brains at E11.5 and afterwards suggests that PCD is probably not a major mechanism for controlling diversity while postmitotic neurons are being generated.

Since apoptotic pathways may affect normal forebrain growth by regulating the expansion of the progenitor population, it is also possible that disturbances in normal developmental apoptosis may lead to congenital brain malformations, such as the reduced number of neurons in the Down syndrome forebrain (Sylvester, 1983; Ross *et al.*, 1984; Wisniewski *et al.*, 1984). For example, increased caspase-mediated cell death in the progenitor population before neurogenesis could abnormally decrease the eventual cortical neuron population. Similarly, reduced protection of neuronal progenitors during neurogenesis by the Jnk pathway could limit eventual cortical growth. Regardless, fully understanding how apoptosis controls cortical growth in individuals and during evolution requires future work delineating the features which make certain progenitors more susceptible to death than others as well as how the various apoptosis pathways may work together.

Notes

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