

# Programmed Cell Death Is a Universal Feature of Embryonic and Postnatal Neuroproliferative Regions Throughout the Central Nervous System

ANNE J. BLASCHKE,<sup>1</sup> JOSHUA A. WEINER,<sup>2</sup> AND JEROLD CHUN<sup>2,3\*</sup>

<sup>1</sup>Department of Biology, School of Medicine, University of California-San Diego, La Jolla, California 92093-0636

<sup>2</sup>Neurosciences Graduate Program, School of Medicine, University of California-San Diego, La Jolla, California 92093-0636

<sup>3</sup>Biomedical Sciences Graduate Program and Department of Pharmacology, School of Medicine, University of California-San Diego, La Jolla, California 92093-0636

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## ABSTRACT

During central nervous system (CNS) development, programmed cell death (PCD) has been viewed traditionally as a fate reserved for differentiating neurons that are in the process of making synaptic connections. Recent studies in the embryonic cerebral cortex (Blaschke et al. [1996] *Development* 122:1165–1174), however, have shown that many neuroblasts in the proliferative ventricular zone undergo PCD as well and that this likely represents a novel form distinct from that found in regions of postmitotic neurons. To determine the commonality of this form of PCD throughout the CNS, the prevalence of dying cells identified by in situ end labeling plus (ISEL+; Blaschke et al. [1996]) was determined within populations of proliferating neuroblasts that were identified by rapid bromodeoxyuridine incorporation. Based on this approach, dying cells were observed to be a common feature of all proliferative neuroblast populations examined. In addition, when ISEL+ was combined with in situ hybridization for *postmitotic neural gene-1* (*png-1*; Weiner and Chun [1997] *J. Comp. Neurol.* 381:130–142), which identifies newly postmitotic neurons, a positive correlation was found between the start of differentiation and the onset of PCD. These data indicate that PCD in neuroblast proliferative zones is a universal feature of nervous system development. Moreover, cell death represents a prominent cell fate that may be linked to mechanisms of differentiation. *J. Comp. Neurol.* 396:39–50, 1998. © 1998 Wiley-Liss, Inc.

**Indexing terms:** cortical development; ventricular zone; apoptosis; neurogenesis; in situ end labeling plus

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The generation of the mammalian cerebral cortex is a complex process that involves the proliferation of neurons in an anatomically defined region, the ventricular zone (Boulder Committee, 1970), followed by the migration of newly postmitotic neurons to a superficial location, where they differentiate and make synaptic connections (for review, see Bayer and Altman, 1991). After each cell division, neuroblasts in the proliferative region have been thought classically to “decide” between one of two fates: continued proliferation or differentiation (Bayer and Altman, 1991). However, theoretically, there is a third fate that could befall neurons in the ventricular zone: They could undergo programmed cell death (PCD). Recent data indicate that many cortical neuroblasts in the proliferative ventricular zone undergo PCD (Blaschke et al., 1996).

Because these neuroblasts have not yet made synaptic connections, this PCD is likely to represent a novel form different from that associated with synaptic competition. The existence of a proliferative form of PCD has received experimental support through the generation of mice

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A.J.B. and J.A.W. contributed equally to this paper.

\*Correspondence to: Jerold Chun, The Department of Pharmacology, School of Medicine, University of California-San Diego, 9500 Gilman Drive, La Jolla, CA 92093-0636. E-mail: jchun@ucsd.edu

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deficient for the pro-cell death *ced-3* homologue gene *CPP32* (Kuida et al., 1996), which resulted in mice with marked hyperplasia throughout the early embryonic central nervous system (CNS) that was not attributable to uncontrolled cell proliferation. The defect was obvious in the cerebral cortex even by embryonic day 12 (E12), an age that precedes the generation of cortical neurons that are found in the adult (Gillies and Price, 1993; Price et al., 1997).

The phenotype of the *CPP32* *-/-* mouse along with prior data from other systems indicate that PCD in proliferative regions may not be restricted to the embryonic cortex. For example, PCD has been observed in proliferative regions of the chick spinal cord (Homma et al., 1994). Retroviral studies of the adult mouse subventricular zone have indicated that, among proliferating cells, the number of cells per clone is rarely greater than one or two, despite multiple cell divisions associated with increased survival time after infection (Morshead and van der Kooy, 1992), suggesting that some progeny were eliminated by PCD.

To determine the commonality of proliferative zone PCD, bromodeoxyuridine (BrdU) immunohistochemistry, in situ end labeling plus (ISEL+; Blaschke et al., 1996; Chun and Blaschke, 1997), and ligation-mediated polymerase chain reaction (LMPCR; Chun and Blaschke, 1997; Staley et al., 1997) were combined to analyze representative proliferative regions throughout the embryonic murine neural tube as well as postnatal proliferative regions. In addition, in situ hybridization for a novel marker gene, *png-1*, which identifies young neurons soon after they become postmitotic (Weiner and Chun, 1997), has allowed the discrimination of postmitotic and proliferative regions in the CNS during development. By using these approaches, we demonstrate that proliferative zone PCD is a universal feature of the CNS with a developmental onset that is coordinated both temporally and spatially with neuronal differentiation.

## MATERIALS AND METHODS

### Tissue collection and preparation

All animal protocols have been approved by the Animal Subjects Committee at the University of California, San Diego, and conform to National Institutes of Health (NIH) guidelines and public law. A total of 38 embryonic and 30 postnatal/adult mice were examined. Tissue sections were prepared as described previously (Chun et al., 1991; Blaschke et al., 1996). Timed-pregnant Balb/c mice (Simonsen, Gilroy, CA) were killed by cervical dislocation. Embryos were removed rapidly, embedded in Tissue-Tek (Fisher Scientific, Pittsburgh, PA), and frozen on dry ice. Early postnatal mice were killed by swift decapitation. Desired tissues were removed and embedded as described above. Sections were cut at 10  $\mu$ m (for ISEL+) or 20  $\mu$ m (for *png-1* in situ hybridization) on a cryostat (Frigocut 2800E; Jung, Nussloch, Germany) and collected onto charged slides. Sections were fixed in 4% paraformaldehyde, extracted with 0.6% Triton X-100 (Sigma, St. Louis, MO) in 2  $\times$  sodium saline phosphate EDTA buffer (SSPE; 2  $\times$  SSPE = 300 mM NaCl, 20 mM NaH<sub>2</sub>PO<sub>4</sub> · H<sub>2</sub>O, 25 mM EDTA, pH 7.4), and acetylated. Sections were dehydrated through graded ethanols and used immediately or were stored at  $-80^{\circ}$ C.

### Detection of programmed cell death: ISEL+

ISEL+ was performed as described previously (Blaschke et al., 1996). Sections (10  $\mu$ m thick) were incubated for 1 hour in labeling mix (100 mM potassium cacodylate, 2 mM CoCl<sub>2</sub>, 0.2 mM dithiothreitol [DTT], 0.5  $\mu$ M digoxigenin-11-dUTP; Boehringer-Mannheim, Indianapolis, IN) containing 125 U/ml terminal transferase (TdT; Boehringer-Mannheim). Incorporated digoxigenin-11-dUTP was detected by an overnight incubation with alkaline phosphatase-conjugated antidigoxigenin Fab fragments (Boehringer-Mannheim) at a dilution of 1:500. Alkaline phosphatase activity was detected by incubation in substrate solution (100 mM Tris, 100 mM NaCl, 50 mM MgCl<sub>2</sub>, pH 9.5) containing 450  $\mu$ g/ml 4-nitroblue tetrazolium chloride (NBT; Boehringer-Mannheim) and 17.5  $\mu$ g/ml 5-bromo-4-chloro-3-indolyl phosphate (BCIP; Boehringer-Mannheim). Color was allowed to develop for 1–2 hours. After washing, nuclei were stained fluorescently by incubating sections in 4,6-diamino-2-phenylindole (DAPI; Sigma) at a concentration of 0.35  $\mu$ g/ml for 15 minutes. Sections were rinsed in Milli-Q H<sub>2</sub>O, mounted in Crystal Mount (Biomedica Corp., Foster City, CA), and photographed by using a Zeiss microscope (Thornwood, NY).

### Detection of cell proliferation

Proliferating cells were detected by using the thymidine analogue BrdU (Sigma). Timed-pregnant Balb/c mice, postnatal mice, or adult mice were injected i.p. with 20  $\mu$ l/g body weight of 10 mM BrdU in saline. After a 1-hour survival (timed-pregnant/postnatal mice) or repeated injections over a 24-hour period (adult mice), animals were killed and sectioned as described above. Sections were incubated overnight at 65°C in a solution of 2  $\times$  standard saline citrate (SSC; 1  $\times$  SSC = 150 mM NaCl, 15 mM Na<sub>3</sub> citrate · 2H<sub>2</sub>O, pH 7.0)/50% formamide to denature DNA, washed in 2  $\times$  SSC, and incubated for 30 minutes in 2 N HCl at 37°C. Sections were then neutralized in 0.1 M boric acid, pH 8.5, washed in phosphate-buffered saline (PBS), and blocked for 1 hour in 2.5% bovine serum albumin (BSA)/0.3% Triton X-100/PBS block, pH 7.4. Anti-BrdU antibody (Boehringer-Mannheim) was added at a concentration of 6  $\mu$ g/ml in blocking solution and was allowed to bind overnight. Sections were washed in PBS, and bound anti-BrdU antibody was detected by using an avidin biotin complex-horseradish peroxidase (ABC-HRP) kit (Vector Laboratories, Burlingame, CA) and 3,3'-diaminobenzidine (DAB; Sigma) as the chromogen. Color was allowed to develop for 5–15 minutes. Sections were dehydrated through graded ethanols and xylene, mounted in Cytoseal (Stephens Scientific, Riverdale, NJ), and photographed by using a Zeiss microscope.

### Double-labeling with BrdU and ISEL+

Double labeling was performed by integrating the ISEL+ and BrdU detection protocols to show unequivocally that proliferation and PCD occur together. Sections were end labeled according to the ISEL+ protocol and then transferred to 2  $\times$  SSC/50% formamide at 65°C overnight before incubation with antidigoxigenin Fab fragments. Sections were washed in 2  $\times$  SSC, and the ISEL+ protocol was completed through colorimetric detection (see above). Sections were then incubated in 2 N HCl, neutralized, and BrdU was detected by immunohistochemistry as described above. To document the relationship between BrdU-

immunostained cells and ISEL+ labeling with low-magnification photography, consecutive sections were photographed, which allows improved resolution compared with double-labeled materials. Figure 1 was composed from scanned photographic negatives in Adobe Photoshop 4.0 (Adobe Systems, Mountain View, CA) on an Apple Macintosh computer (Apple Computers, Cupertino, CA). Only minor color tone corrections were made before printing the figure on an Epson Stylus Color 800 printer (Nagano, Japan).

### DNA isolation

DNAs were isolated by rapid dissection of tissue and lysis in digestion buffer (75 mM NaCl, 25 mM EDTA, 10 mM Tris, pH 8.0, 1% sodium dodecyl sulfate, 0.4 mg/ml freshly added proteinase K). After overnight digestion, DNAs were phenol/chloroform extracted, precipitated, spooled onto glass rods, dried, and dissolved in 10 mM Tris and 1 mM EDTA, pH 8.0, and quantified by using a spectrophotometer.

### LMPCR

LMPCR was performed as described previously (Staley et al., 1997). Briefly, 6 µg genomic DNA were mixed with 1 nmol 24 base pairs (bp) and 1 nmol 12 bp unphosphorylated oligonucleotides, annealed, and ligated. Ligations were stored at -20°C until PCR was performed. PCR reactions used approximately 150 ng ligated DNA and used the 24-bp oligonucleotide as a primer. Samples were amplified for 25 cycles of PCR, and PCR products were analyzed by agarose gel electrophoresis.

Control PCR for the single-copy gene *engrailed-2* (*en-2*) was performed to ensure equal loading of DNA in LMPCR (Staley et al., 1997). *En-2* PCR used amounts of ligated DNA identical to those used for LMPCR. Samples were amplified for 28 cycles of PCR.

### *Png-1* in situ hybridization

Digoxigenin-labeled *png-1* sense and antisense riboprobes were transcribed from a plasmid containing the full open reading frame by using standard protocols (Genius System; Boehringer-Mannheim). Tissue sections were prepared as described above, and in situ hybridization was performed as described previously (Chun et al., 1991; Hecht et al., 1996; Weiner and Chun, 1997). Briefly, sections were hybridized overnight at 65°C with sense or antisense riboprobe at a concentration of 2 ng/µl in situ hybridization buffer (50% formamide, 2 × SSPE, 10 mM dithiothreitol, 2 mg/ml yeast tRNA, 0.5 mg/ml polyadenylic acid, 2 mg/ml BSA, 0.5 mg/ml denatured salmon sperm DNA). Sections were washed twice for 45 minutes each at room temperature in 2 × SSPE/0.6% Triton X-100, then washed three times for 30 minutes each at 65°C in 1 × high-stringency buffer (2 mM Na<sub>4</sub>P<sub>2</sub>O<sub>7</sub>, 1 mM Na-free EDTA, 1 mM Na<sub>2</sub>HPO<sub>4</sub>). Sections were rinsed in 1 × Tris-buffered saline, blocked for 1 hour in 1% blocking solution (Boehringer-Mannheim)/0.3% Triton X-100, and incubated overnight in a 1:500 dilution of alkaline phosphatase-conjugated antidigoxigenin Fab fragments (Boehringer-Mannheim). Alkaline phosphatase activity was detected by incubation in substrate solution like that used for ISEL+ (see above).

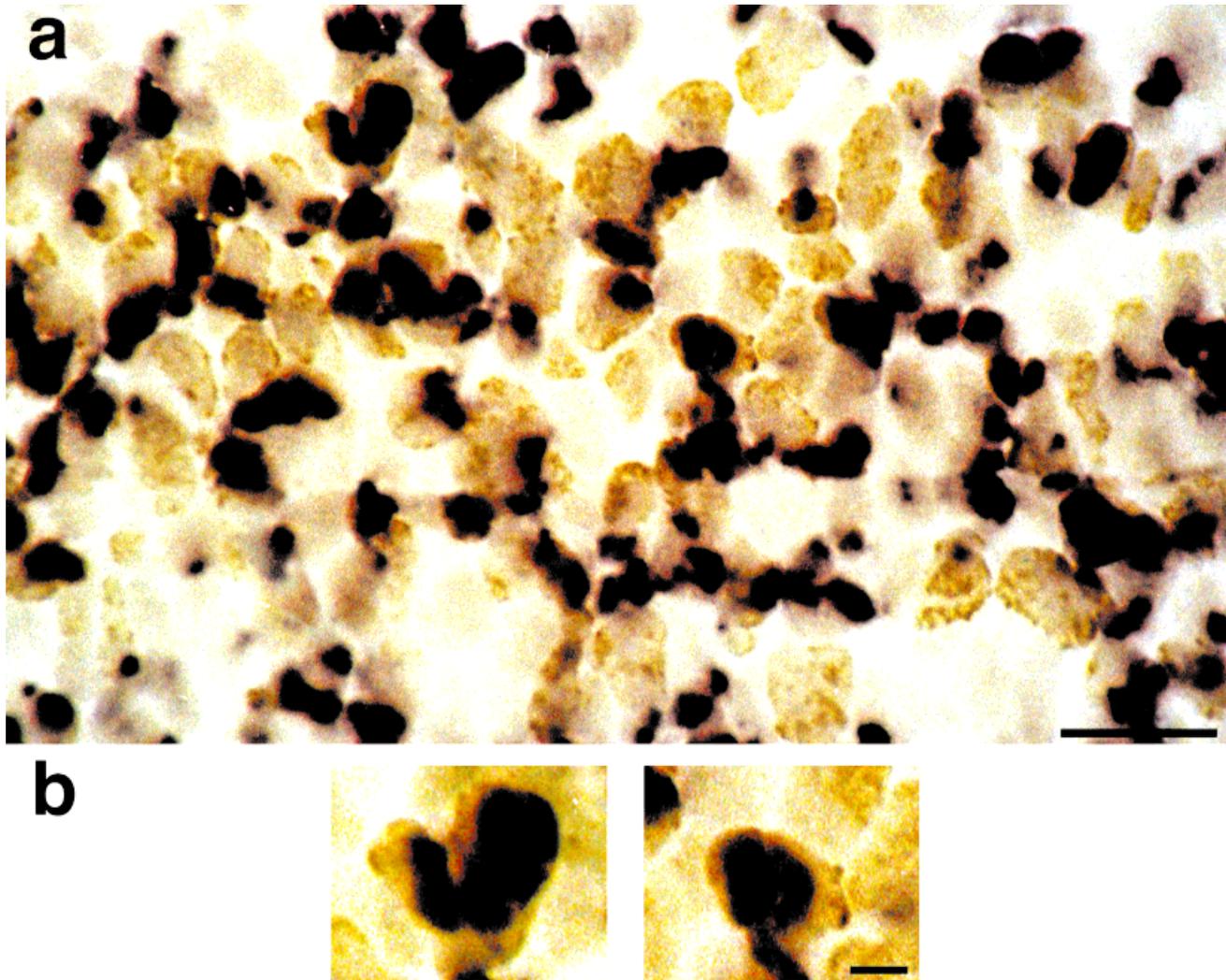
## RESULTS

To determine whether PCD in proliferative regions is a general feature of nervous system development, ISEL+ (Blaschke et al., 1996) was combined with identification of proliferating neuroblasts throughout the developing CNS by using BrdU incorporation and immunohistochemistry. In previous studies, ISEL+ was shown to be specific in identifying cells undergoing PCD (Blaschke et al., 1996; Chun and Blaschke, 1997). ISEL+ identifies dying cells in both in vivo and in vitro model systems of PCD from both neural and nonneural tissues, including the dexamethasone-treated thymus and the early postnatal retina, and there is a specific increase in ISEL+-labeled cells over controls when PCD is induced experimentally. ISEL+ is dependent on the activity of the terminal deoxynucleotidyl transferase enzyme, which attaches labeled nucleotides to the free ends of fragmented DNA in dying cells; however, ISEL+ does not label the Okazaki fragments of cells undergoing DNA synthesis (e.g., see Fig. 6). Furthermore, ISEL+ detection of dying cells correlates precisely and positively with the presence of apoptotic nucleosomal ladders detected by LMPCR (Blaschke et al., 1996; Chun and Blaschke, 1997; Staley et al., 1997).

Pregnant mice were injected i.p. with BrdU on E12 or E14 and were killed 1 hour later: This brief pulse of BrdU labels cells in S phase at the superficial border of the ventricular zone. Embryos were removed, sectioned, and processed for ISEL+ and/or BrdU immunohistochemistry on the same section or on alternating adjacent tissue sections. Double-labeled sections were examined to confirm the colocalization of proliferation and PCD. An example from the developing cerebral cortex at E14 is shown in Figure 1. In this high-magnification view of the cortical S-phase zone, BrdU+ cells are tan, whereas ISEL++ cells are purple-black (Fig. 1a). Cells double labeled for ISEL+ and BrdU (shown at greater magnification in Fig. 1b) were common in all regions of the CNS examined at both E12 and E14. BrdU was administered to embryos only 1 hour before being killed; therefore, the presence of both BrdU and ISEL+ staining in the same cell suggested that it had undergone DNA synthesis and had entered the PCD pathway within the last hour.

The prevalence of PCD within proliferating regions throughout the developing nervous system was surveyed on E12 and E14, around the midpoint of the neurogenic period (Gardette et al., 1982), as well as in the early postnatal animal and the adult (see Tables 1 and 2). Representative regions from the cerebral cortex (Fig. 2a-c), thalamus (Fig. 2d-f), hindbrain (Fig. 2g-i) and spinal cord (Fig. 2j-l) were examined by using ISEL+, BrdU immunostaining, and DAPI fluorescence on adjacent sections or on the same section. In each case, neuroproliferation that had been identified by a 1-hour pulse of BrdU delineated regions within which many ISEL+-labeled cells could be identified. Other embryonic structures (not shown; see Table 2) in which ISEL+-labeled cells were detected within proliferative regions included the olfactory bulb, tectum, cerebellar primordium, trigeminal ganglion, and dorsal root ganglia.

Regions in which neuroblasts proliferate during the early postnatal period (Sidman, 1961; Altman and Das, 1965; Fujita et al., 1966; Altman and Bayer, 1978; Stanfield and Cowan, 1979; Lois and Alvarez-Buylla, 1993; Luskin, 1993) were also examined by using combined



**Fig. 1.** Proliferating cells identified by bromodeoxyuridine (BrdU) incorporation can undergo programmed cell death (PCD) in the embryonic central nervous system (CNS). Pregnant females carrying embryonic day 14 (E14) mouse embryos were pulsed with BrdU 1 hour prior to killing, and embryo sections were double-labeled for in situ end-labeling plus (ISEL+) and BrdU immunohistochemistry. **a:** A high-magnification view of the S-phase zone (superficial ventricular zone) of the cerebral cortex at E14 is shown. In this and in all other

CNS regions examined, cells labeled by ISEL+ (purple-black) are found in significant numbers among those identified as proliferating by BrdU immunostaining (tan). A number of cells appear to be double-labeled by both ISEL+ and BrdU immunohistochemistry. **b:** Two further magnified views of double-labeled cells. Double-labeling in this experiment suggests that some cells were in S phase within 1 hour before initiating DNA fragmentation. Scale bars = 20  $\mu\text{m}$  in a, 4  $\mu\text{m}$  in b.

**ISEL+/BrdU labeling.** The retinal photoreceptor layer (Fig. 3a–c), the dentate gyrus of the hippocampus (Fig. 3d–f), and the external granule layer of the cerebellum (Fig. 3g–i) all contained proliferating cells at postnatal day 3 (P3). Consistent with findings in the embryo, ISEL+ identified many cells undergoing PCD within each proliferating region. Cells undergoing PCD, as noted previously by others (Thomaidou et al., 1997), were also detected in the early postnatal subventricular zone (not shown; see Table 2).

The subventricular zone is a region of neuroblast proliferation that persists into the adult period (Morshead and van der Kooy, 1992; Gage et al., 1995), and data have supported the occurrence of PCD within this zone during adulthood based on the small clone sizes observed in retroviral lineage studies (Morshead and van der Kooy,

1992). Combined ISEL+/BrdU examinations demonstrated that many cells in the adult subventricular zone are labeled by ISEL+ (Fig. 4a). The spatial pattern of ISEL+ labeling overlaps with that of BrdU immunostaining on an adjacent tissue section (Fig. 4b), consistent with the occurrence of PCD within this adult neuroproliferative region.

To confirm that ISEL+ labeling was representative of cells undergoing PCD, LMPCR detection of the nucleosomal ladders associated with apoptosis (Chun and Blaschke, 1997; Staley et al., 1997) was performed on genomic DNA from selected proliferative regions. LMPCR is an independent, semiquantitative assay, and DNA laddering identified by LMPCR is always proportional to labeling observed by using ISEL+ (Blaschke et al., 1996; Chun and Blaschke, 1997; Staley et al., 1997). Genomic DNA was

TABLE 1. Structures Examined in This Study and Their Reported Period of Neuroproliferation

Region	Structures examined	Period of neuron generation <sup>1</sup>	References
Forebrain	Cerebral cortex	E10–E18	Angevine and Sidman, 1961 Caviness and Sidman, 1973
	Ganglionic eminence	E10–E16	Sidman and Angevine, 1962
	Olfactory bulb	E10–E18	Hinds, 1968
	Hippocampus	E12 to 1 year	Angevine, 1965 Stanfield and Cowan, 1979
	Subventricular zone	E15 to adult	Morshead and van der Kooy, 1992 Luskin, 1993
	Thalamus	E10–E16	Johnston and Angevine, 1966 Angevine, 1970
Midbrain	Tectum	E10–E18	Delong and Sidman, 1962
Hindbrain	Cerebellum-Purkinje/ Golgi cells	E11–E16	Altman and Bayer, 1978
	Granule cells	E17–P10	Fujita et al., 1966
	Pons	E11–E14	Altman and Bayer, 1980b
	Medulla	E10–E14	Altman and Bayer, 1980a
Spinal cord		E10–E14	Nornes and Das, 1974 Nornes and Carry, 1978
Peripheral nervous system	Dorsal root ganglia	E10–E14	Lawson and Biscoe, 1979
	Trigeminal ganglion	E10–E14	Forbes and Welt, 1981
Retina	Photoreceptor cells	E13–P6	Sidman, 1961

<sup>1</sup>E, embryonic day; P, postnatal day.

TABLE 2. Age and Number of Animals and Structures Examined in This Study

Age	Number of animals examined	Structures examined
E12–E14	38	Cerebral cortex Ganglionic eminence Olfactory bulb Tectum Thalamus Cerebellum-Purkinje cells Pons Medulla Trigeminal ganglion Dorsal root ganglia Spinal cord
P3	10	Subventricular zone Hippocampus Retina-photoreceptor cells Cerebellum-granule cells Subventricular zone
Adult	20	

isolated from the forebrain, midbrain, and hindbrain of the embryo and from the P3 retina and was analyzed by LMPCR (Fig. 5). In these samples and in all others from neuroproliferative regions (data not shown), LMPCR identified apoptotic DNA ladders, indicating that ISEL+ indeed recognizes cells undergoing apoptosis.

A consistent result in these and previous studies of the cerebral cortex (Blaschke et al., 1996; Kuida et al., 1996) was that the initiation of PCD did not occur until the start of postmitotic neuron generation after E10 (Berry and Rogers, 1965; Gillies and Price, 1993). This suggested a link between PCD and the initiation of neuroblast differentiation. To examine this possibility, the known spatial and temporal patterns of neuronal differentiation in the cerebral cortex and spinal cord (Nornes and Carry, 1978; Smart and Smart, 1982; Bayer and Altman, 1991) were

examined by combining ISEL+ with in situ hybridization for a zinc-finger gene that has been shown recently to identify newly postmitotic neurons, *postmitotic neural gene-1* (*png-1*; Weiner and Chun, 1997).

The embryonic cerebral cortex (Fig. 6a–d) and spinal cord (Fig. 6h–k) were first examined at ages known to precede the generation of postmitotic neurons. Despite the high level of cell proliferation, as detected by BrdU immunostaining (Fig. 6c,j), very little ISEL+ labeling was observed (Fig. 6a,h). Note that this absence of ISEL+ labeling despite the high level of BrdU immunostaining demonstrates that Okazaki fragments are not labeled by ISEL+, and, furthermore, that the employed dosage of BrdU did not in itself induce PCD. *Png-1* expression was not detected on adjacent sections (Fig. 6b,i), consistent with data, which indicate that postmitotic neuron production had not yet begun in the E10 cortex (Weiner and Chun, 1997) or the E9 spinal cord (Nornes and Carry, 1978). Subsequently, *png-1* expression in each of these areas was detectable (1–2 days later) as a thin rim of cells at the superficial surface of the neural tube, corresponding to the first postmitotic neurons to migrate out of the underlying ventricular zone (Fig. 6f,m, arrows). The timing of this expression coincided with the initiation of significant PCD, as detected by ISEL+ (Fig. 6e,l). These data demonstrate a temporal correlation between the production and migration of the first postmitotic neurons and the onset of neuroproliferative PCD in both the cortex and the spinal cord.

To examine this phenomenon further, the known spatial gradient of cortical neuron differentiation (Smart and Smart, 1982; Bayer and Altman, 1991) was examined. In the embryonic cortex, postmitotic neuron generation begins up to 1 day earlier ventrolaterally than dorsomedially, leading to a decreasing gradient of postmitotic neurons from ventrolateral regions to dorsomedial regions (Smart and Smart, 1982). Consistent with the temporal correlation between the onset of PCD and *png-1* expression shown above, there was also a positive spatial correlation between the ventrolateral-dorsomedial gradient of differentiation (demonstrated by *png-1* labeling; see Fig. 7a) and the gradient of neuroproliferative PCD (Fig. 7b). This correlation was confirmed by quantification (Fig. 7d). These temporal and spatial correlations together suggest that the onset of PCD in a given ventricular zone is linked to the initiation of postmitotic neuron generation in that ventricular zone.

## DISCUSSION

Prior studies on the ventricular zone have shown that PCD is a significant cell fate for developing cortical neuroblasts (Blaschke et al., 1996; Staley et al., 1997). To determine the generality of this phenomenon, we have extended these studies to examine neuroproliferative regions throughout the CNS by combining brief pulses of BrdU to identify actively proliferating populations of neuroblasts with ISEL+ and LMPCR for the identification of dying cells. Regions of proliferation and PCD were found to overlap extensively along the length of the neural tube during embryonic development, and this relationship continued in regions of postnatal neuroblast proliferation. Moreover, by combining ISEL+ with in situ hybridization for the novel zinc-finger transcription factor gene *png-1* (Weiner and Chun, 1997), a temporal and spatial correla-

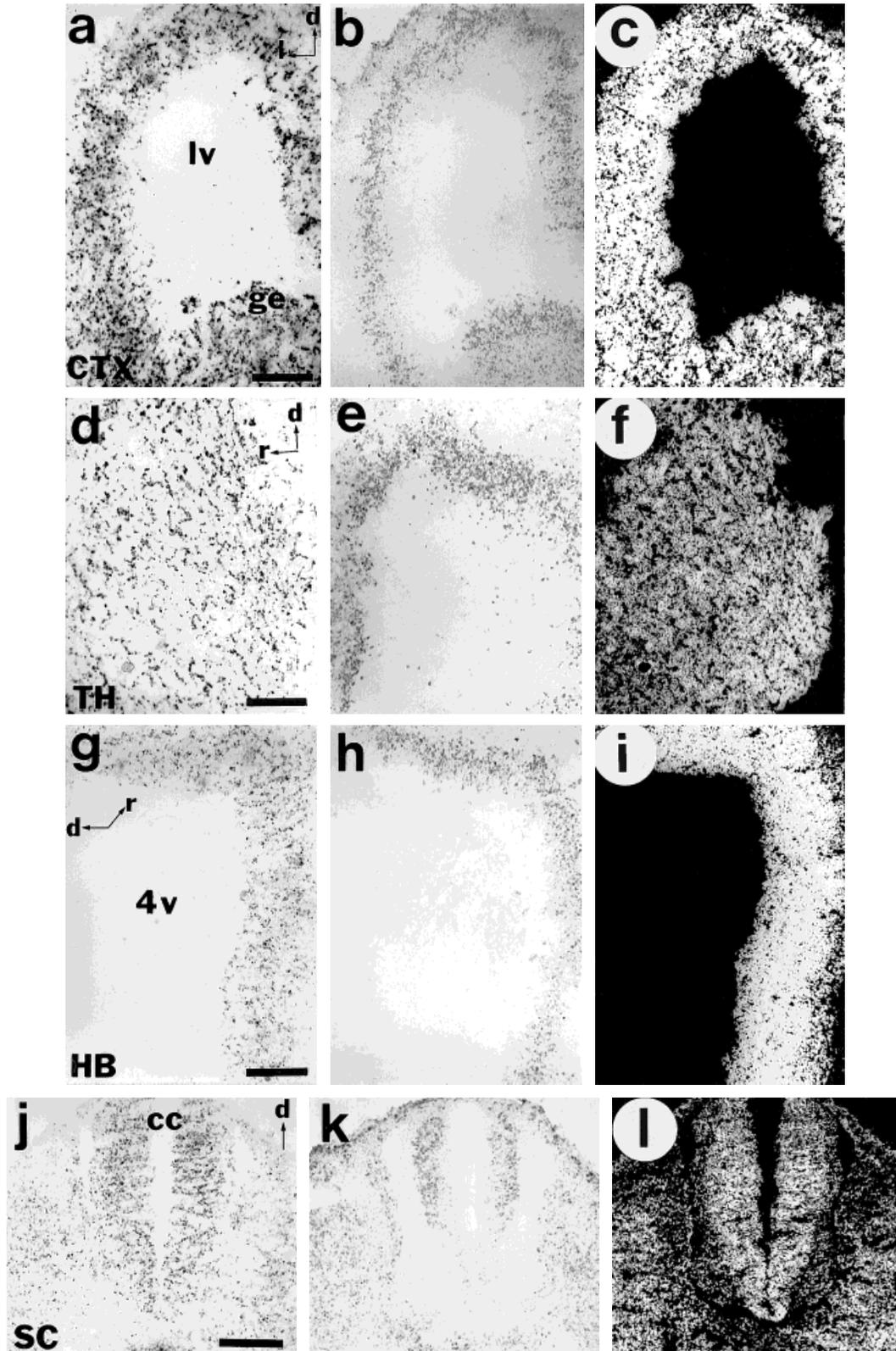


Fig. 2. Cells undergoing programmed cell death (PCD) are found in proliferative regions throughout the embryonic central nervous system (CNS). Adjacent sections from embryonic day 12 (E12; g-l) or E14 (a-f) mice that were pulsed in utero for 1 hour with bromodeoxyuridine (BrdU) were processed for BrdU immunohistochemistry (b,e,h,k) to identify regions of cell proliferation or for in situ end-labeling plus (ISEL+) staining (a,d,g,j) to identify cells undergoing PCD. 4,6-Diamino-2-phenylindole (DAPI) fluorescence of the ISEL+-labeled section in each row (c,f,i,l) serves as a comparative counterstain. The proliferative regions are defined at their superficial extents by the BrdU+ cells; their inner extents are at the ventricular surface.

a-c: Coronal section through the E14 cerebral cortex (CTX) is shown for comparison (see Blaschke et al., 1996). Many cells in the ventricular zone are undergoing PCD. ISEL+-labeled cells are also detected in the proliferative ganglionic eminence (ge). d-f: Sagittal section through the E14 thalamus (TH). g-i: Sagittal section through the E12 hindbrain (HB; future pons and medulla). j-l: Coronal section through the E12 spinal cord (SC). The hindbrain and spinal cord develop earlier than the forebrain and thalamus; therefore, they are shown at E12. d, dorsal; l, lateral; r, rostral; lv, lateral ventricle; 4v, fourth ventricle; cc, central canal. Scale bar = 200  $\mu$ m.

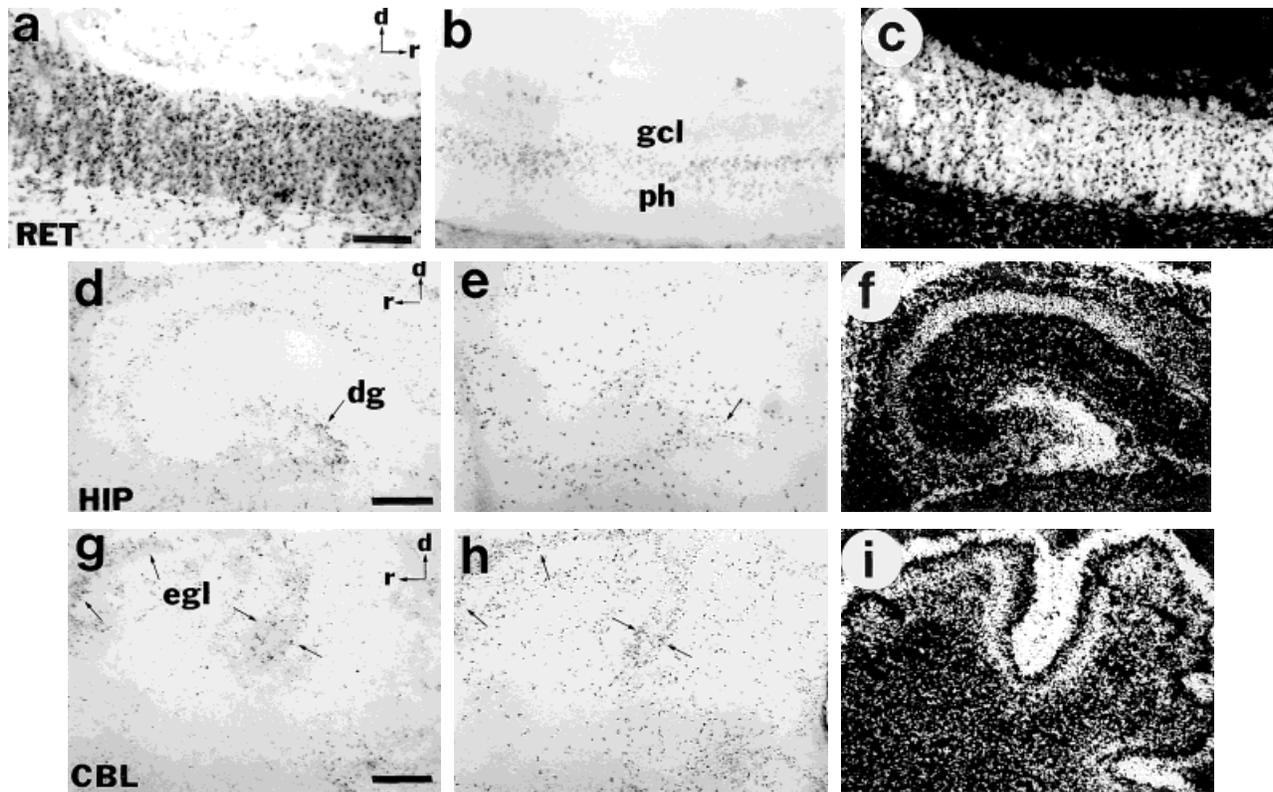


Fig. 3. Cells undergoing programmed cell death (PCD) are found in proliferative regions of postnatal central nervous system (CNS) structures. Several regions of the CNS maintain neuroproliferation during the early postnatal period. These regions were examined in sagittal sections of postnatal day 3 (P3) mice that were pulsed for 1 hour with bromodeoxyuridine (BrdU). ISEL<sup>+</sup> staining (a,d,g) was combined with BrdU immunohistochemistry on an adjacent section (b,e,h), and 4,6-Diamino-2-phenylindole (DAPI) fluorescence of the in situ end-labeling plus (ISEL<sup>+</sup>) section (c,f,i). a–c: Neurons in the photoreceptor layer (ph) of the mouse retina (RET) are proliferating at this age, and many of these cells are also labeled by ISEL<sup>+</sup>. ISEL<sup>+</sup>-labeled cells are

also seen throughout the postmitotic ganglion cell layer (gcl), which is known to contain dying cells at this age as well. d–f: Granule neurons in the dentate gyrus (dg) of the hippocampus (HIP) are also proliferating at P3 (e), and some are ISEL<sup>++</sup> (d). g–i: The external granule cell layer (egl) of the cerebellum (CBL) produces neurons that migrate to populate the internal granule cell layer of the adult. Here, too, ISEL<sup>+</sup>-labeled cells are observed (g) in the proliferating regions defined by BrdU incorporation (h). Arrows in d,e and g,h mark the same regions, respectively. d, Dorsal; r, rostral. Scale bars = 100 μm in a (also applies to b,c), 200 μm in d (also applies to e,f) and g (also applies to h,i).

tion between the initial differentiation of neurons and the start of PCD was documented. Together, these data indicate that PCD is a significant cell fate for proliferating neuroblasts throughout the neural tube and that this fate is likely to be linked to the process of neuronal differentiation.

In studies of the embryonic mammalian nervous system, cell death has been noted on occasion to occur at low levels in neuroproliferative regions of the CNS. These observations relied on histological or electron micrographic studies (Glucksmann, 1951; Saunders, 1966; Silver, 1978) and accounted for only a few percent of cells, an insignificant amount compared with the substantial cell death associated with postmitotic neurons undergoing synapse formation (Hamburger and Oppenheim, 1982; Lance-Jones, 1982; Oppenheim, 1985). In contrast, recent studies using techniques that detect apoptotic DNA have given a markedly different view of the prevalence of PCD in neuroproliferative regions (Blaschke et al., 1996; Chun and Blaschke, 1997; Staley et al., 1997; Thomaidou et al., 1997). For example, in the cortical ventricular zone, an average of approximately 50% of cells can be detected by ISEL<sup>+</sup> over the period of cortical neurogenesis (Blaschke et al., 1996).

ISEL<sup>+</sup> labeling is also associated with the fragmentation of DNA into “nucleosomal ladders,” which are characteristic of apoptosis (Wyllie, 1980), as detected by LMPCR (Blaschke et al., 1996; Staley et al., 1997). Results obtained here in various CNS proliferative regions parallel those reported for the cortical ventricular zone (Blaschke et al., 1996). Several other reports have described PCD in nonmammalian neuroproliferative regions, such as those of *C. elegans* (Hedgecock et al., 1983; Ellis and Horvitz, 1986) and the embryonic chick spinal cord (Homma et al., 1994). The occurrence of neuroproliferative PCD throughout the CNS observed here, along with its operation in nonmammalian organisms, indicates that this form of PCD may represent a general feature of CNS development throughout phylogeny.

An important but still unresolved issue is the determination of the absolute extent of PCD that occurs in the neural tube. In the studies presented here, as in all studies using ISEL<sup>+</sup> or related DNA end-labeling techniques, the clearance time of labeled cells is impossible to determine with certainty: The process of labeling (e.g., fixation of tissue) prevents one from following the subsequent elimination of a labeled cell. To follow individual cells labeled by ISEL<sup>+</sup>

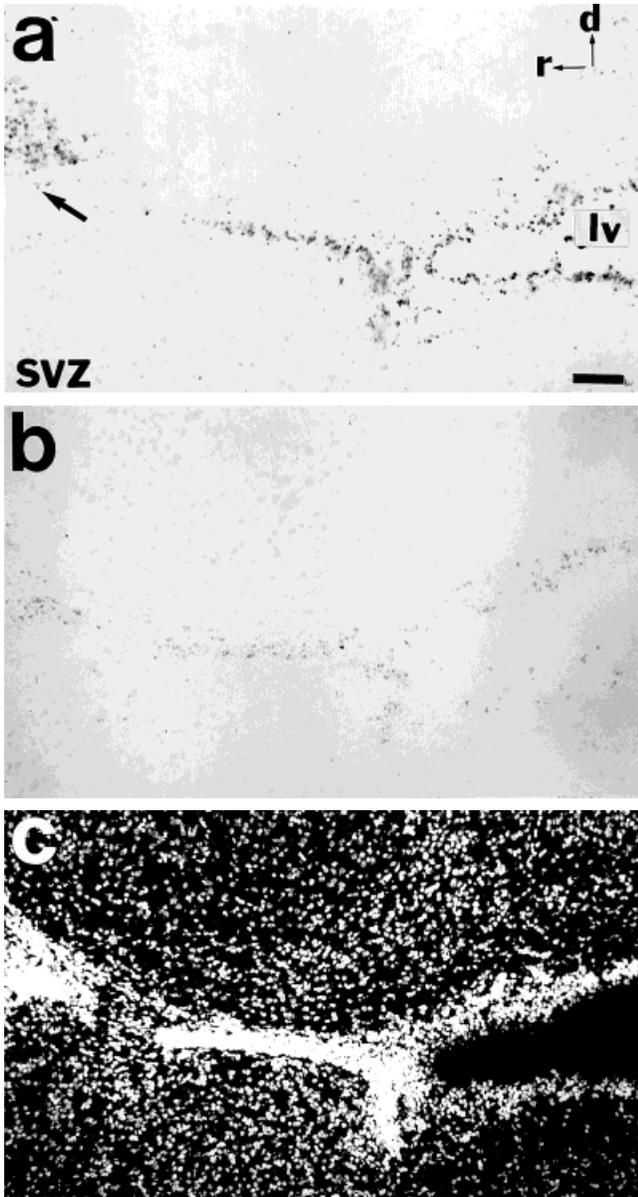


Fig. 4. **a-c:** Programmed cell death (PCD) is a prominent fate for cells proliferating in the adult subventricular zone (SVZ). The subventricular zone is a region of the adult rodent central nervous system (CNS) in which neuroproliferation occurs. Neurons in the anterior part of this region travel along the rostral migratory stream to populate the olfactory bulb. Repeated pulses of bromodeoxyuridine (BrdU) were given to adult mice over a 24-hour period before killing and tissue processing. In situ end-labeling plus (ISEL+) labeling (a) detects dying cells in a pattern that overlaps with that of BrdU immunohistochemistry on an adjacent sagittal section (b), indicating that many of the cells born in the adult subventricular zone undergo PCD before leaving this region. c: 4,6-Diamino-2-phenylindole (DAPI) fluorescence of the section in shown in a. The arrow in a indicates the direction in which neurons of the rostral migratory stream migrate into the olfactory bulb. d, Dorsal; lv, lateral ventricle; r, rostral. Scale bar = 100  $\mu$ m.

in real time is the only way to determine unequivocally the period from initial labeling through elimination, but this is not currently practical. By using less sensitive morphologi-

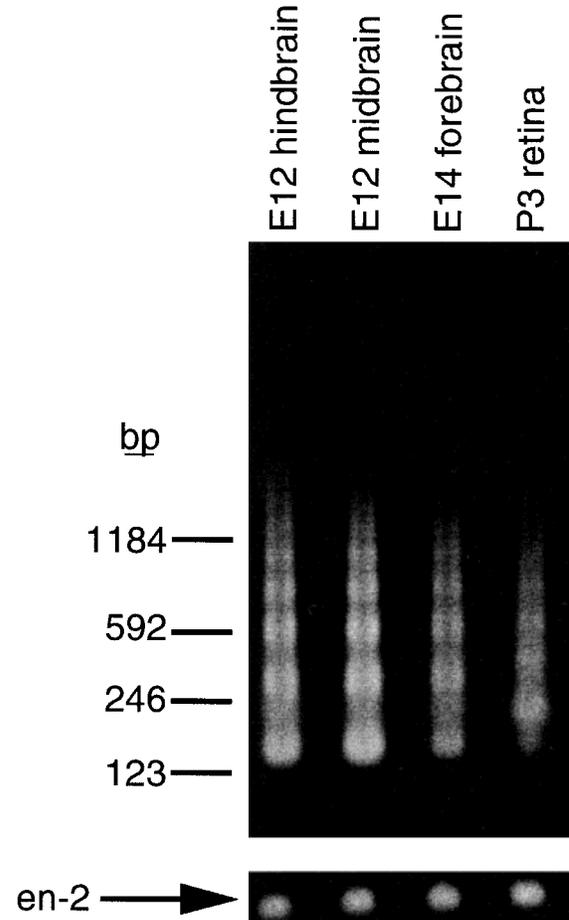


Fig. 5. Ligation-mediated polymerase chain reaction (LMPCR) analysis of DNA from embryonic and postnatal proliferative regions confirms the occurrence of programmed cell death (PCD). LMPCR is a sensitive assay for the detection of "nucleosomal ladders" that result from DNA fragmentation accompanying apoptosis (Staley et al., 1997). Genomic DNA was isolated from the forebrain, midbrain, and hindbrain of embryonic mice during proliferative periods as well as from the P3 retina. LMPCR (25 cycles) amplified apoptotic ladders from all of these regions as well as from other proliferative regions of the perinatal mouse (e.g., E12 spinal cord, P3 cerebellum; data not shown). The detection of apoptotic DNA fragmentation by using LMPCR is consistent with the operation of PCD in these proliferative regions. PCR for the engrailed-2 gene (*en-2*) was used as a loading control. bp, Base pairs.

cal techniques, the clearance time of apoptotic cells is thought to be in the range of hours (Barres et al., 1992; Raff et al., 1993), which may be viewed as the minimum time required to eliminate a DNA end-labeled cell (Gavrieli et al., 1992; Blaschke et al., 1996; Chun and Blaschke, 1997): The maximum time required remains unknown. By using a mammalian model of cell death, the small intestinal vilus, in which cells turn over completely every 2-4 days, it is clear that at least some cells commence apoptotic DNA fragmentation, as detected by ISEL+, up to several days before their actual elimination (Pompeiano et al., 1998). This extended latency between the onset of DNA fragmentation and the elimination of the cell is consistent with studies of the retina, in which dying cells identified by less sensitive morphological methods were estimated to

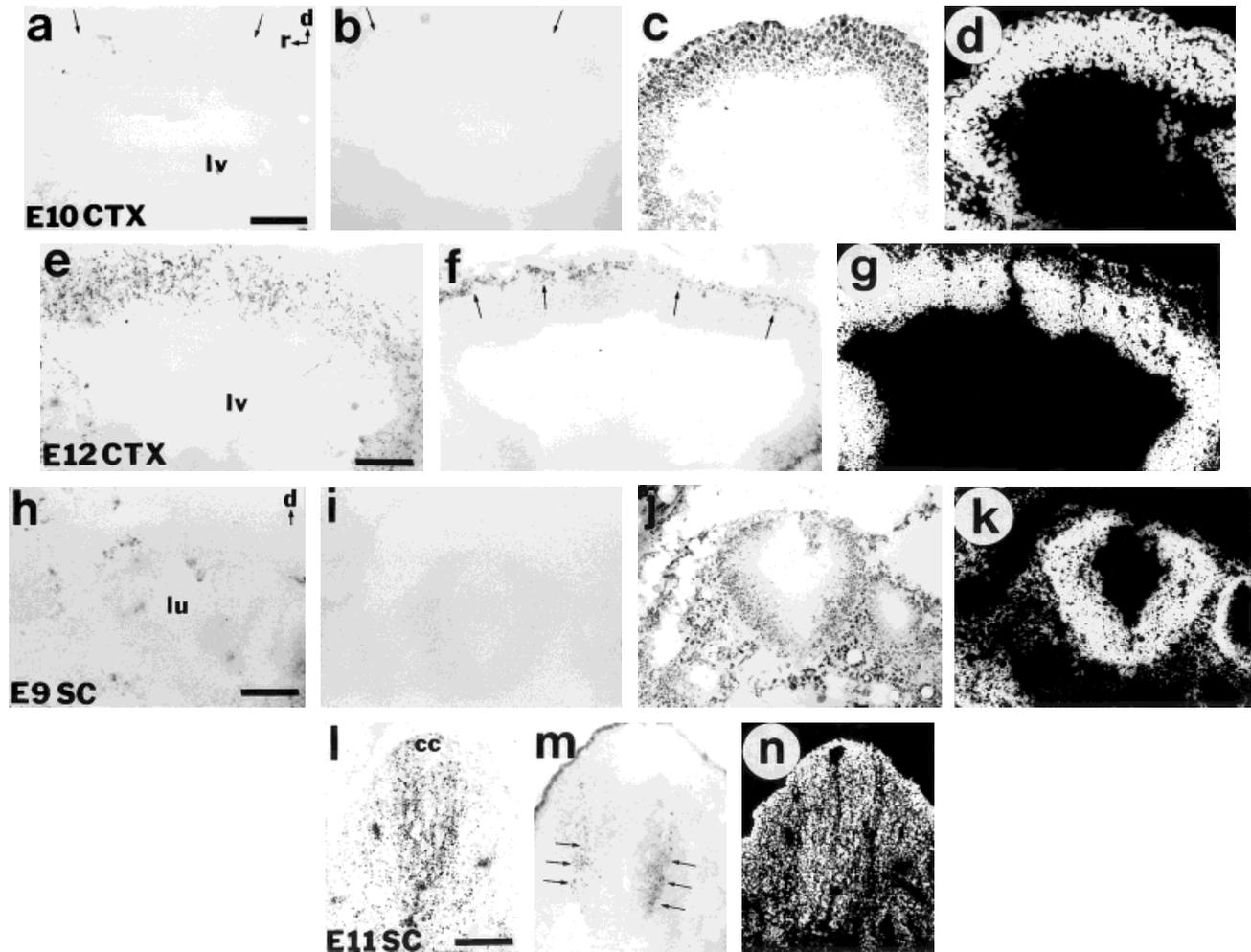


Fig. 6. **a-n**: Programmed cell death (PCD) onset is linked temporally to the initiation of neuroblast differentiation. Cells undergoing PCD were identified by in situ end-labeling plus (ISEL+; a,e,h,l), and postmitotic neurons were identified by in situ hybridization for *png-1*, a zinc-finger gene that is expressed by newly postmitotic neurons (Weiner and Chun, 1997), on adjacent sections (b,f,i,m). In sagittal sections through the presumptive cerebral cortex (CTX) at E10 (a-d) and in coronal sections through the future spinal cord (SC) at E9 (h-k), neurons have not yet begun to differentiate (Nornes and Carry, 1978; Bayer and Altman, 1991), as confirmed by the absence of *png-1* in situ hybridization signal (b,i). These regions also contain few cells that have started to undergo PCD (a,h) despite exhibiting a high degree of bromodeoxyuridine (BrdU) incorporation in response to a 1-hour pulse (c,j). Note further the absence of ISEL+ labeled cells at the early ages

(a,h) despite this high degree of BrdU immunostaining, indicating that Okazaki fragments are not labeled by ISEL+ and, moreover, that the employed dosage of BrdU does not in itself induce PCD. Two days later in both the cortex (f) and the spinal cord (m), *png-1* in situ hybridization identifies postmitotic neurons as a thin rim of cells near the outer surface of the neural tube (arrows in f and m). The presence of these early postmitotic neurons, which have migrated superficially after being generated in the ventricular zone, coincides with the onset of widespread ISEL+ labeling in the ventricular zone itself (e,l), indicating a temporal link between the onset of neuroproliferative PCD and the generation of the first postmitotic neurons. (d,g,k,n) DAPI fluorescence of ISEL+ section. Arrows in a and b mark the outer edge of the cortex. lv, Lateral ventricle; lu, lumen of the neural tube; cc, central canal. Scale bars = 200  $\mu$ m.

remain for 24–48 hours (Miller and Oberdorfer, 1981; Cunningham, 1982; Horsburgh and Sefton, 1987). In addition, recent studies have shown that dying cells identified by morphological criteria remain in the adult hippocampus for up to 3 days before elimination following an apoptotic stimulus (Hu et al., 1997). In view of these data, we estimate that clearance times of ISEL+ labeled cells range from about 1 hour up to days. We suggest, therefore, that, although all ISEL+ labeled cells are dying and will be eliminated, only a fraction of these cells will be eliminated on a given day and that the total population of ISEL+ labeled cells is a mix of cells commencing, midway through, or near the end of the DNA fragmentation compo-

nent of their death program. The mathematical function describing this distribution is not known, but, by using a Gaussian distribution as a hypothetical example, the population of eliminated cells could be a small percentage of the total number at any given time point. The total number of cells ultimately fated to die in various CNS regions remains to be determined, but results from a mouse mutation of CPP32/Caspase-3/apopain/YAMA hint at the relative magnitude (Kuida et al., 1996).

CPP32 is a pro-cell death interleukin-1- $\beta$  converting enzyme (ICE)-like protease that is the mammalian gene most homologous to *C. elegans ced-3* (Fernandes-Alnemri et al., 1994; Xue et al., 1996). The CPP32<sup>-/-</sup> mice displayed

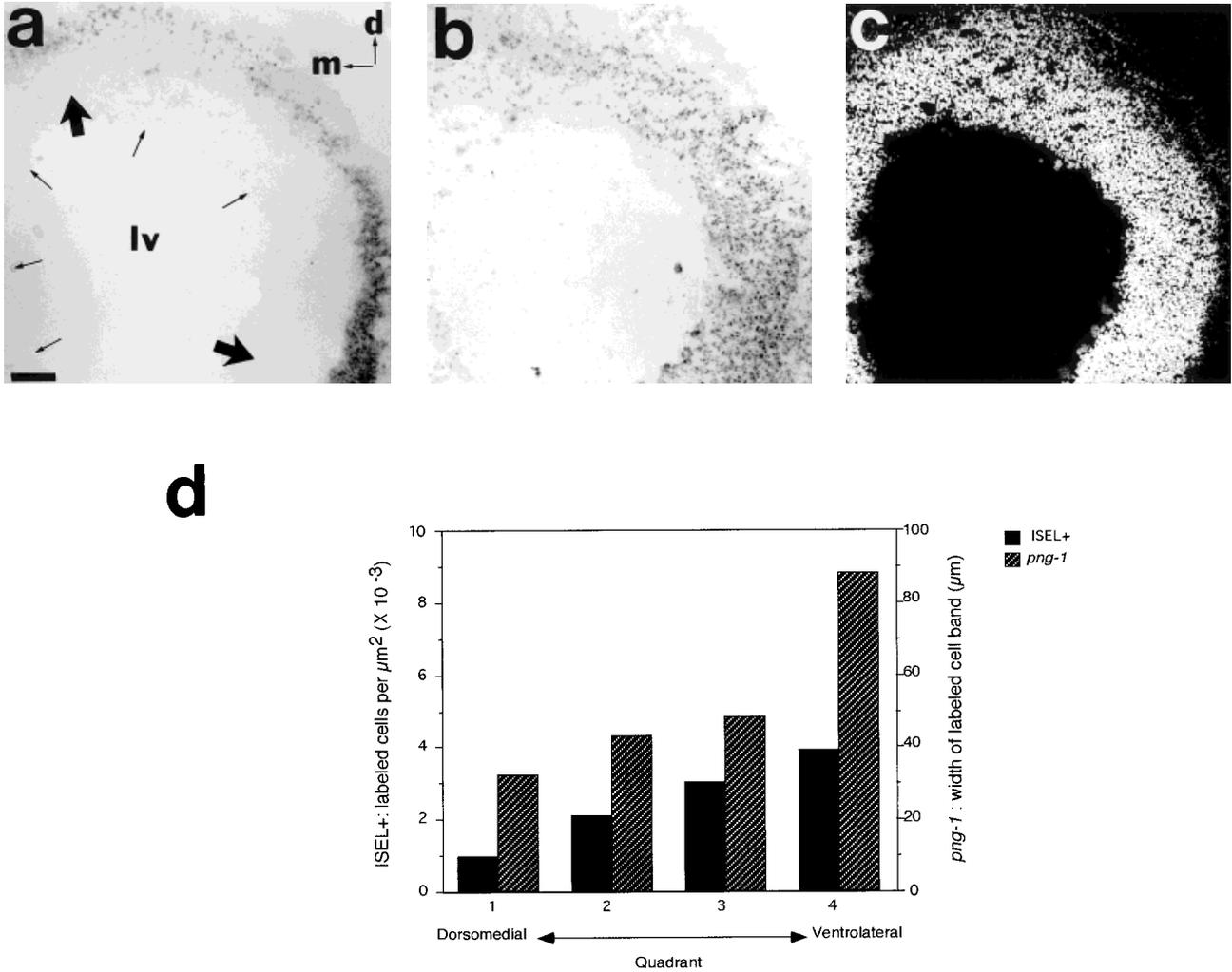


Fig. 7. A spatiotemporal gradient of programmed cell death (PCD) in the embryonic cortex parallels the ventrolateral-dorsomedial gradient of neuronal differentiation. **a:** *Png-1* in situ hybridization was used to demonstrate the ventrolateral-dorsomedial gradient of differentiation within the cerebral cortex at E12 (Bayer and Altman, 1991) in a coronal section. Note the comparatively thick superficial band of *png-1*<sup>+</sup> neurons in the lateral portion of the cortex, which thins toward the medial portion. **b:** In situ end-labeling plus (ISEL<sup>+</sup>) identification of dying cells in an adjacent section of cortex shows a similar gradient. There are many more cells identified by ISEL<sup>+</sup> in the lateral portion of the cortex than near the midline. **c:** 4,6-Diamino-2-phenylindole (DAPI) fluorescence of the section shown in **a**. **d:** The sections shown in

**a** and **b** were divided into four equal quadrants from dorsomedial to ventrolateral (between the thick arrows in **a**) for quantification. Measurements were taken from photographic prints of each quadrant at a magnification of  $\times 630$ . The number of ISEL<sup>+</sup>-labeled cells/ $\mu\text{m}^2$  in each quadrant was plotted along with a determination of the average width ( $\mu\text{m}$ ) of the superficial band of *png-1*<sup>+</sup> neurons. Quadrant 1 is the most dorsomedial quadrant, and quadrant 4 is most ventrolateral. The increase in *png-1*<sup>+</sup> neurons is paralleled by the increase in ISEL<sup>+</sup>-labeled cells, illustrating the correlation between gradients of differentiation and of PCD. Thin arrows in **a** mark the borders of the lateral ventricle (lv). d, Dorsal; m, medial. Scale bar = 100  $\mu\text{m}$ .

supernumerary cells throughout the early neural tube, which could not be attributed to oncogenic increases in proliferation (Kuida et al., 1996). The presence of these extra cells is likely the result of decreased PCD (Kuida et al., 1996), particularly within the proliferative population (A.J. Blaschke, J.A. Weiner, R. Flavell, and J. Chun, unpublished observations). The phenotype of the CPP32 null mouse is so significant that it causes a severalfold increase in the thickness of the neural tube by E12 (Kuida et al., 1996) as well as aberrant foldings within cortical regions (A.J. Blaschke, J.A. Weiner, R. Flavell, and J. Chun, unpublished observations). This extent of supernumerary cell production before the majority of postmitotic

neurons have been generated (Weiner and Chun, 1997) is consistent with the extensive operation of PCD in the early embryonic cortex (Blaschke et al., 1996) and in other regions of the embryonic CNS observed here. We note that no published estimates of the extent of neuroproliferative PCD can account for the CPP32<sup>-/-</sup> phenotype aside from those obtained by using ISEL<sup>+</sup>.

Other studies are also consistent with the operation of extensive neuroproliferative PCD. In the early postnatal mouse subventricular zone, TdT-mediated dUTPnick end labeling (TUNEL) reveals extensive PCD (Thomaidou et al., 1997), and our data are consistent with these observations. Retroviral labeling studies of early cortical neuro-

blasts (Austin and Cepko, 1990; Acklin and van der Kooy, 1993; Cai et al., 1997) and adult subventricular zone cells (Morshead and van der Kooy, 1992) consistently report clone sizes of one or two cells, far fewer than would be expected in the absence of PCD based on estimated cell cycle times (Caviness et al., 1995). Taken together, our results and those of prior studies indicate that neuroproliferative PCD occurs throughout the mammalian CNS.

The cellular and molecular mechanisms underlying neuroproliferative PCD are not known, but they likely represent a form of PCD distinct from that occurring among mature neurons making connections with their adult synaptic targets. Although the biochemical mechanisms involved might be the same, synaptogenesis has not begun in most of the proliferative regions noted here; therefore, by definition, cells in these regions cannot be undergoing synaptic, "target-dependent" PCD. We speculate on three possible elements that may participate in initiating neuroproliferative PCD. One is a limited quantity of established neurotrophic factors or expression of cognate receptors. For example, it has been shown that endogenous nerve growth factor expressed in the embryonic chick retina can cause PCD of retinal neurons, but only if they express the p75 neurotrophin receptor (Frade et al., 1996). A second possible element encompasses nontraditional neural growth factors. Within the cerebral cortex, a candidate molecule is the growth factor-like lipid lysophosphatidic acid (LPA). An LPA receptor gene, *vzg-1*, was isolated recently and was found to be expressed in the ventricular zone of the cerebral cortex (Hecht et al., 1996), which places it at the correct time and location to mediate at least some signaling aspects of cortical PCD. A third possible element is a process analogous to that observed in the developing thymus, in which T cells are selected specifically by thymic stromal cells based on expression of certain molecules (Shortman et al., 1990; Surh and Sprent, 1994). The actual mechanisms responsible remain to be determined.

A new facet to such mechanisms is the finding that neuroproliferative PCD does not become pronounced until differentiating postmitotic cells are also present, based on the use of the novel marker *png-1* (Weiner and Chun, 1997). This correlation suggests a link between the onset of PCD in a given ventricular zone and the initial generation of postmitotic neurons from that ventricular zone, although the nature of this link is unclear. Based on the observation that postmitotic cell fate (at least in the cortex) appears to be determined during a neuroblast's final S phase (McConnell and Kaznowski, 1991), a possible explanation is that this fate determination can lead subsequently to one of two outcomes: either mitosis and the production of one or two postmitotic neurons, or death commencing at or around S phase (Ninomiya et al., 1997). This is surely an incomplete view of the actual interactions, which remain to be determined. However, these data are consistent with the postulate that some form of cell selection occurs in these neuroproliferative regions (Blaschke et al., 1996) and that PCD begins only when selective mechanisms—perhaps associated with differentiation—are activated. Future analyses will determine the precise mechanisms through which neuroproliferative PCD is controlled and the influence of PCD on CNS cell lineage, fate, and proliferation kinetics.

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## LITERATURE CITED

- Acklin, S.E. and D. van der Kooy (1993) Clonal heterogeneity in the germinal zone of the developing rat telencephalon. *Development* **118**:175–192.
- Altman, J. and S.A. Bayer (1978) Prenatal development of the cerebellar system in the rat. *J. Comp. Neurol.* **179**:23–48.
- Altman, J. and S.A. Bayer (1980a) Development of the brainstem in the rat. II. Thymidine-radiographic study of the time of origin of neurons of the vestibular and auditory nuclei of the upper medulla. *J. Comp. Neurol.* **194**:877–904.
- Altman, J. and S.A. Bayer (1980b) Development of the brainstem in the rat. V. Thymidine-radiographic study of the time of origin of neurons in the pontine region. *J. Comp. Neurol.* **194**:905–929.
- Altman, J. and G.D. Das (1965) Autoradiographic and histological evidence of postnatal hippocampal neurogenesis in rats. *J. Comp. Neurol.* **124**:319–336.
- Angevine, J.B. (1970) Time of neuron origin in the diencephalon of the mouse. An autoradiographic study. *J. Comp. Neurol.* **139**:129–188.
- Angevine, J.B. (1965) Time of neuron origin in the hippocampal region. An autoradiographic study in the mouse. *Exp. Neurol.* **2**(Suppl.):1–70.
- Angevine, J.B. and R.L. Sidman (1961) Autoradiographic study of cell migration during histogenesis of cerebral cortex in the mouse. *Nature* **192**:766–768.
- Austin, C.P. and C.L. Cepko (1990) Cellular migration patterns in the developing mouse cerebral cortex. *Development* **110**:713–732.
- Barres, B.A., I.K. Hart, H.S. Coles, J.F. Burne, J.T. Voyvodic, W.D. Richardson, and M.C. Raff (1992) Cell death and control of cell survival in the oligodendrocyte lineage. *Cell* **70**:31–46.
- Bayer, S.A. and J. Altman (1991) *Neocortical Development*. New York: Raven Press, Ltd.
- Berry, M. and A.W. Rogers (1965) The migration of neuroblasts in the developing cerebral cortex. *J. Anat.* **99**:691–709.
- Blaschke, A.J., K. Staley, and J. Chun (1996) Widespread programmed cell death in proliferative and postmitotic regions of the fetal cerebral cortex. *Development* **122**:1165–1174.
- Boulder Committee (1970) Embryonic vertebrate central nervous system: Revised terminology. *Anat. Rec.* **166**:257–261.
- Cai, L., N.L. Hayes, and R.S. Nowakowski (1997) Synchrony of clonal cell proliferation and contiguity of clonally related cells: Production of mosaicism in the ventricular zone of developing mouse neocortex. *J. Neurosci.* **17**:2088–2100.
- Caviness, V.S.J. and R.L. Sidman (1973) Time of origin of corresponding cell classes in the cerebral cortex of normal and reeler mutant mice: An autoradiographic analysis. *J. Comp. Neurol.* **148**:141–152.
- Caviness, V.S.J., T. Takahashi, and R.S. Nowakowski (1995) Numbers, time and neocortical neurogenesis: A general developmental and evolutionary model. *Trends Neurosci.* **18**:379–383.
- Chun, J. and A.J. Blaschke (1997) Identification of neural programmed cell death through the detection of DNA fragmentation in situ and by PCR. In J.N. Crawley, C.R. Gerfen, R. McKay, M.A. Rogawski, D.R. Sibley, and P. Skolnick (eds): *Current Protocols In Neuroscience*. New York: John Wiley and Sons, pp. 3.8.1–3.8.19.
- Chun, J.J.M., D.G. Schatz, M.A. Oettinger, R. Jaenisch, and D. Baltimore (1991) The recombination activating gene-1 (RAG-1) transcript is present in the murine central nervous system. *Cell* **64**:189–200.
- Cunningham, T.J. (1982) Naturally occurring neuron death and its regulation by developing neural pathways. *Int. Rev. Cytol.* **74**:163–186.
- Delong, G.R. and R.L. Sidman (1962) Effects of eye removal at birth on histogenesis of mouse superior colliculus. An autoradiographic analysis with tritiated thymidine. *J. Comp. Neurol.* **118**:205–221.
- Ellis, H.M. and H.R. Horvitz (1986) Genetic control of programmed cell death in the nematode *C. elegans*. *Cell* **44**:817–829.

- Fernandes-Alnemri, T., G. Litwack, and E.S. Alnemri (1994) CPP32, a novel human apoptotic protein with homology to *Caenorhabditis elegans* cell death protein Ced-3 and mammalian interleukin-1 $\beta$ -converting enzyme. *J. Biol. Chem.* *269*:30761–30764.
- Forbes, D.J. and C. Welt (1981) Neurogenesis in the trigeminal ganglion of the albino rat: A quantitative autoradiographic study. *J. Comp. Neurol.* *199*:133–147.
- Frade, J.M., A. Rodriguez-Tebar, and Y. Barde (1996) Induction of cell death by endogenous nerve growth factor through its p75 receptor. *Nature* *383*:166–168.
- Fujita, S., M. Shimada, and T. Nakamura (1966) H3-thymidine autoradiographic studies on the cell proliferation and differentiation in the external and the internal granular layers of the mouse cerebellum. *J. Comp. Neurol.* *128*:191–208.
- Gage, F.H., J. Ray, and L.J. Fisher (1995) Isolation, characterization and use of stem cells from the CNS. *Annu. Rev. Neurosci.* *18*:159–192.
- Gardette, R., M. Courtois, and J.-C. Bisconte (1982) Prenatal development of mouse central nervous structures: Time of neuron origin and gradients of neuronal production. A radioautographic study. *J. Hirnforsch* *23*:415–431.
- Gavrieli, Y., Y. Sherman, and S.A. Ben-Sasson (1992) Identification of programmed cell death in situ via specific labeling of nuclear DNA fragmentation. *J. Cell. Biol.* *119*:493–501.
- Gillies, K. and D.J. Price (1993) The fates of cells in the developing cerebral cortex of normal and methylazoxymethanol acetate-lesioned mice. *Eur. J. Neurosci.* *5*:73–84.
- Glucksmann, A. (1951) Cell deaths in normal vertebrate ontogeny. *Biol. Rev.* *26*:59–86.
- Hamburger, V. and R.W. Oppenheim (1982) Naturally-occurring neuronal death in vertebrates. *Neurosci. Comm.* *1*:38–55.
- Hecht, J.H., J.A. Weiner, S.R. Post, and J. Chun (1996) Ventricular zone gene-1 (*vzgf-1*) encodes a lysophosphatidic acid receptor expressed in neurogenic regions of the developing cerebral cortex. *J. Cell. Biol.* *135*:1071–1083.
- Hedgecock, E.M., J.E. Sulston, and J.N. Thomson (1983) Mutations affecting programmed cell deaths in the nematode *Caenorhabditis elegans*. *Science* *220*:1277–1279.
- Hinds, J.W. (1968) Autoradiographic study of histogenesis in the mouse olfactory bulb: Time of origin of neurons and neuroglia. *J. Comp. Neurol.* *134*:287–304.
- Homma, S., H. Yaginuma, and R.W. Oppenheim (1994) Programmed cell death during the earliest stages of spinal cord development in the chick embryo: A possible means of early phenotypic selection. *J. Comp. Neurol.* *345*:377–395.
- Horsburgh, G.M. and A.J. Sefton (1987) Cellular degeneration and synaptogenesis in the developing retina of the rat. *J. Comp. Neurol.* *263*:553–566.
- Hu, Z., K. Yuri, H. Ozawa, H. Lu, and M. Kawata (1997) The in vivo time course for elimination of adrenalectomy-induced apoptotic profiles from the granule cell layer of the rat hippocampus. *J. Neurosci.* *17*:3981–3989.
- Johnston, A.M. and J.B. Angevine (1966) Autoradiographic study of neuron origin in the diencephalon in the mouse. *Anat. Rec.* *154*:363.
- Kuida, K., T.S. Zheng, S. Na, C. Kuan, D. Yang, H. Karasuyama, P. Rakic, and R.A. Flavell (1996) Decreased apoptosis in the brain and premature lethality in CPP32-deficient mice. *Nature* *384*:368–372.
- Lance-Jones, C. (1982) Motoneuron cell death in the developing lumbar spinal cord of the mouse. *Dev. Brain Res.* *4*:473–479.
- Lawson, S.N. and T.J. Biscoe (1979) Development of mouse dorsal root ganglia: An autoradiographic and quantitative study. *J. Neurocytol.* *8*:265–274.
- Lois, C. and A. Alvarez-Buylla (1993) Proliferating subventricular zone cells in the adult mammalian forebrain can differentiate into neurons and glia. *Proc. Natl. Acad. Sci. USA* *90*:2074–2077.
- Luskin, M.B. (1993) Restricted proliferation and migration of postnatally generated neurons derived from the forebrain subventricular zone. *Neuron* *11*:173–189.
- McConnell, S.K. and C.E. Kaznowski (1991) Cell cycle dependence of laminar determination in the developing neocortex. *Science* *254*:282–285.
- Miller, N. and M. Oberdorfer (1981) Neuronal and neuroglial responses following retinal lesions in the neonatal rats. *J. Comp. Neurol.* *202*:493–504.
- Morshead, C.M. and D. van der Kooy (1992) Postmitotic death is the fate of constitutively proliferating cells in the subependymal layer of the adult mouse brain. *J. Neurosci.* *12*:249–256.
- Ninomiya, Y., R. Adams, G.M. Morriss-Kay, and K. Eto (1997) Apoptotic cell death in neuronal differentiation of P19 EC cells: Cell death follows reentry into S phase. *J. Cell. Physiol.* *172*:25–35.
- Nornes, H.O. and M. Carry (1978) Neurogenesis in spinal cord of mouse: An autoradiographic analysis. *Brain Res.* *159*:1–16.
- Nornes, H.O. and G.D. Das (1974) Temporal pattern of neurogenesis in spinal cord of rat. I. An autoradiographic study—Time and sites of origin and migration and settling patterns of neuroblasts. *Brain Res.* *73*:121–138.
- Oppenheim, R.W. (1985) Naturally occurring cell death during neural development. *Trends Neurosci.* *8*:487–493.
- Pompeiano, M., M. Hvala, and J. Chun (1998) Onset of apoptotic DNA fragmentation can precede cell elimination by days in the small intestinal villus. *Cell Death Diff.* (in press).
- Price, D.J., S. Aslam, L. Tasker, and K. Gillies (1997) Fates of the earliest generated cells in the developing murine neocortex. *J. Comp. Neurol.* *377*:414–422.
- Raff, M.C., B.A. Barres, J.F. Burne, H.S. Coles, Y. Ishizaki, and M.D. Jacobson (1993) Programmed cell death and the control of cell survival: Lessons from the nervous system. *Science* *262*:695–700.
- Saunders, J.W.J. (1966) Death in embryonic systems. *Science* *154*:604–612.
- Shortman, K., M. Egerton, G.J. Spangrude, and R. Scollay (1990) The generation and fate of thymocytes. *Semin. Immunol.* *2*:3–12.
- Sidman, R.L. (1961) Histogenesis of mouse retina studied with thymidine-H3. In G.K. Smelser (ed): *The Structure of the Eye*. New York: Academic Press, pp. 487–506.
- Sidman, R.L. and J.B. Angevine (1962) Autoradiographic analysis of time of origin of nuclear vs. cortical components of mouse telencephalon. *Anat. Rec.* *142*:326–327.
- Silver, J. (1978) Cell death during development of the nervous system. In M. Jacobson (ed): *Handbook of Sensory Physiology*. Vol. IX: Development of Sensory Systems. Berlin: Springer-Verlag, pp. 419–436.
- Smart, I.H.M. and M. Smart (1982) Growth patterns in the lateral wall of the mouse telencephalon: I. Autoradiographic studies of the histogenesis of the isocortex and adjacent areas. *J. Anat.* *134*:273–298.
- Staley, K., A.J. Blaschke, and J. Chun (1997) Apoptotic DNA fragmentation is detected by a semi-quantitative ligation-mediated PCR of blunt DNA ends. *Cell. Death Diff.* *4*:66–75.
- Stanfield, B.B. and W.M. Cowan (1979) The development of the hippocampus and dentate gyrus in normal and reeler mice. *J. Comp. Neurol.* *185*:423–460.
- Surh, C.D. and J. Sprent (1994) T-cell apoptosis detected in situ during positive and negative selection in the thymus. *Nature* *372*:100–103.
- Thomaidou, D., M.C. Mione, J.F.R. Cavanagh, and J.G. Parnavelas (1997) Apoptosis and its relation to the cell cycle in the developing cerebral cortex. *J. Neurosci.* *17*:1075–1085.
- Weiner, J.A. and J. Chun (1997) Png-1, a nervous system-specific zinc finger gene, identifies regions containing postmitotic neurons during mammalian embryonic development. *J. Comp. Neurol.* *381*:130–142.
- Wyllie, A.H. (1980) Glucocorticoid-induced thymocyte apoptosis is associated with endogenous endonuclease activation. *Nature* *284*:555–556.
- Xue, D., S. Shaham, and H.R. Horvitz (1996) The *Caenorhabditis elegans* cell-death protein CED-3 is a cysteine protease with substrate specificities similar to those of the human CPP32 protease. *Genes. Dev.* *10*:1073–1083.