Development/Plasticity/Repair

Essential Role for Survivin in Early Brain Development

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Apoptosis is an essential process during normal neuronal development. Approximately one-half of the neurons produced during neurogenesis die before completion of CNS maturation. To characterize the role of the inhibitor of apoptosis gene, *survivin*, during neurogenesis, we used the Cre-*loxP*-system to generate mice lacking *survivin* in neuronal precursor cells. Conditional deletion of *survivin* starting at embryonic day 10.5 leads to massive apoptosis of neuronal precursor cells in the CNS. Conditional mutants were born at the expected Mendelian ratios; however, these died shortly after birth from respiratory insufficiency, without primary cardiopulmonary pathology. Newborn conditional mutants showed a marked reduction in the size of the brain associated with severe, mutifocal apoptosis in the cerebrum, cerebellum, brainstem, spinal cord, and retina. Caspase-3 and caspase-9 activities in the mutant brains were significantly elevated, whereas bax expression was unchanged from controls. These results show that survivin is critically required for the survival of developing CNS neurons, and may impact on our understanding of neural repair, neural development, and neurodegenerative diseases. Our study is the first to solidify a role for survivin as an antiapoptotic protein during normal neuronal development *in vivo*.

Key words: **survivin; brain development; retina; neuron; embryo; gene deletion; apoptosis; mice**

Introduction

Programmed cell death is a critical process that ultimately defines the number of neurons in the brain and spinal cord required for normal nervous system function during mammalian development (Sastry and Rao, 2000). Survivin is a member of the inhibitor of apoptosis protein (IAP) family by virtue of its structural and functional homology to other IAP members (Ambrosini et al., 1997). Its genomic locus encodes at least five different survivin isoforms, identified in both humans and mice (Mahotka et al., 1999; Conway et al., 2000; Caldas et al., 2005). It is ubiquitously expressed during embryonic development, but only minimally expressed in adult tissues. Interestingly, it is widely expressed in human malignant tumors, making it an exciting target for cancer therapy. Unlike other IAP proteins, survivin plays a dual role as an apoptotic inhibitor and as a chromosomal passenger protein (CPP) (Altieri, 2003).

To date, *in vivo* genetic models of *survivin* deficiency do not uniformly support the role of survivin in apoptosis. *Survivin*-deficient embryos die *in utero* by day 4.5 of gestation, exhibiting disrupted microtubules and polyploidy suggesting defective cytokinesis, without obvious alterations in the number of apoptotic cells (Uren et al., 2000). Targeted disruption of *survivin* within developing T-cells results in defective proliferation and maturation, also without inducing apoptosis (Xing et al., 2004). In contrast to these genetic studies, however, overexpression of survivin can protect human tumor cells from many different forms of apoptosis (Ambrosini et al., 1997; Tamm et al., 1998; Grossman et al., 2001). In addition, disruption of survivin function using antisense, ribozyme, or dominant-negative survivin in both *in vitro* and *in vivo* tumor models induces apoptosis (Ambrosini et al., 1998; Chen et al., 2000; Olie et al., 2000; Pennati et al., 2002, 2004; Pisarev et al., 2003). Thus, the cellular functions of survivin can vary depending on the cell type and cellular context studied.

Survivin is ubiquitously expressed during embryonic development, including the nervous system (Adida et al., 1998), suggesting an important role for this protein in embryogenesis. Within the human brain, we showed previously that it is expressed in regions populated by neural progenitor cells (Altura et al., 2003). Others have confirmed by SAGE (serial analysis of gene expression) that it is one of the most abundant transcripts expressed within neural precursors of the adult mouse brain (Pennartz et al., 2004).

To examine the effects of *survivin* deficiency in neuronal development, we targeted the deletion of *survivin* to neural progenitor cells using a *nestin*-specific promoter expressing *cre-recombinase*. Nestin is an intermediate filament protein considered a marker for neural progenitor cells (Dahlstrand et al., 1995). Through these studies, we show that *survivin* is essential for the survival of developing neurons *in vivo*, thereby underlin-

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ing its central role in a programmed cell death pathway in these cells.

Materials and Methods

Generating mice. Mice harboring a conditionally targeted mouse *survivin* gene flanked by two loxP sites (survivin$^{lox/lox}$) have been described previously (Xing et al., 2004). Male nestin-cre mice, with the cre-recombinase gene under the control of the nestin promoter and a nervous system-specific enhancer (B6.Cg-Tg(nestin-cre)1Kln/J; stock no. 003771; The Jackson Laboratory, Bar Harbor, ME) were crossed with female survivin$^{lox/lox}$ mice. The resulting offspring (nestin-cre; survivin$^{lox/+}$) were backcrossed to survivin$^{lox/lox}$ mice. The morning of vaginal plug detection was designated as embryonic day 0.5 (E0.5). Pregnant dams were killed at different developmental time points (from E9.5 to E19.5). Embryos were dissected and directly observed under the microscope for gross abnormalities. Postnatal day 0 (P0) pups were collected either by cesarean (C)-section or after natural delivery. Pups were closely observed after birth for their general behavior and gross abnormalities. Whole-mount embryo images were obtained using a Leica (Nussloch, Germany) MZ 16FA stereomicroscope equipped with a SPOT RT KE digital camera (Diagnostic Instruments, Sterling Heights, MI). All experimental procedures were performed with the approval of the Institutional Animal Care and Use Committee of Columbus Children's Research Institute. For genotyping, genomic DNA was isolated from mouse tails and yolk sacs using the PureGen kit (Gentra, Minneapolis, MN). Zygosity for the conditional *survivin* mutation was determined by PCR genotyping, as reported previously (Xing et al., 2004). The presence of the nestin-cre transgene was detected by PCR, as described by the The Jackson Laboratory.

Histology, immunohistochemistry, and terminal deoxynucleotidyl transferase-mediated biotinylated UTP nick end labeling staining. Embryos were fixed in either 10% neutral-buffered formalin or Histochoice (Ameresco, Solon, OH), dehydrated through a graded series of ethanol, and embedded in paraffin wax. Sections were cut at 4 μm thickness and stained with hematoxylin and eosin (H&E) for standard histology. Luxol Fast Blue was used to stain myelin. For immunohistochemistry (IHC), the ABC method was used (Fangusaro et al., 2005). The primary antibodies used were as follows: GFAP (1:250; DakoCytomation, Carpinteria, CA), NSE (neuron-specific enolase) (1:750; DakoCytomation), Bax (B-9; 1:50; Santa Cruz Biotechnology, Santa Cruz, CA), active caspase-3 (1:20; Abcam, Cambridge, MA), proliferating cell nuclear antigen (PCNA) (1:100; Upstate Biotechnology, Lake Placid, NY), phosphorylated histone H3 (1:200; Upstate Cell Signaling Solutions, Charlottesville, VA). Secondary antibodies used were biotinylated anti-rabbit IgG (1:200; Santa Cruz Biotechnology) or a Universal Link biotinylated secondary antibody (Biocare Medical, Walnut Creek, CA). Tertiary reagent used was streptavidin-HRP (Biocare Medical). Apoptosis was assayed with the FragEL kit (Calbiochem, La Jolla, CA), according to the manufacturer's instructions. Color was developed using AEC (3-amino-9-ethylcarbazole) chromogen and counterstained with hematoxylin.

To quantitate the differences observed between the mutant and the wild-type littermate brains at E11.5, transverse sections from four knockout and four wild-type embryos were examined at a magnification of 40× by two independent observers (Karanjawala et al., 2002). Neuronal cells staining positive for terminal deoxynucleotidyl transferase-mediated biotinylated UTP nick end labeling (TUNEL), active caspase-3, PCNA, and phosphohistone H3 (PHH3) were manually counted. Five 12,500 μm^2 areas, spanning the full cortical wall, were counted per embryo. The counts were averaged for each embryo, and an independent *t* test with unequal variances was used to test the equality of the groups. A *p* value was considered significant at <0.05.

5-Bromo-2′-deoxyuridine assays. For 5-bromo-2′-deoxyuridine (BrdU), pregnant dams were injected with 100 μg/g BrdU 2 h before killing. Embryos were fixed, as detailed above, and sections were stained with an anti-BrdU antibody (DakoCytomation). Briefly paraffin sections were deparaffinized in xylene and rehydrated in a series of ethanol. Sections were rinsed in PBS, incubated in 200 μg/ml pepsin/10 mM HCl for 20 min at room temperature (RT) followed by incubation in 2N HCl for 45 min. They were neutralized in 0.1 M sodium borate, pH 8.5, for 10 min, washed in PBS, and then blocked with 10% normal goat serum (NGS)/PBS for 1 h at RT, and incubated with anti-BrdU (1:20 in 3% NGS/PBS) at 4°C overnight. After washing in PBS, sections were incubated with anti-mouse-FITC (1:100 in 3% NGS/PBS) for 1 h at RT. Sections were then treated with RNase (100 μg/ml) in 2× SSC for 15 min and counterstained with 500 ng/ml propidium iodide in 2× SSC for 5 min. Sections were then washed, dried, and mounted in Vectashield (Vector Laboratories, Burlingame, CA).

β-Galactosidase assay. To evaluate cre recombinase activity, nestin-cre mice were crossed with B6;129S-Gtrosa26^{tm1sor}, the cre reporter mice (R26R) (Soriano, 1999), and β-galactosidase expression in nestin-cre embryos heterozygous for the cre reporter locus was evaluated at a variety of embryonic stages (E9.5–E14.5). Briefly, embryos were dissected, and then fixed in fresh 4% paraformaldehyde at 4°C for 30 min to 2 h with gentle agitation. Embryos were washed in PBS and stained in 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) staining media (PBS containing 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, 1 mg/ml X-gal, 0.1% deoxycholate, 0.2% NP-40, and 2 mM $MgCl_2$) for 24–48 h at 22–30°C with gentle agitation. Embryos were then washed with PBS and postfixed with either 4% paraformaldehyde or 10% formalin. Photographs for whole-mount staining were taken using a stereomicroscope. For histological examination, embryos were dehydrated through a graded series of ethanol and processed for paraffin embedding. Transverse paraffin sections were cut at 4 μm thickness and counterstained with nuclear fast red.

Caspase assays. Fresh brain tissues dissected from E10.5, E12, E13.5, and E14.5 embryos were homogenized using a Vibra Cell homogenizer (Sonics & Materials, Danbury, CT). Protein concentrations were quantified using Dc Protein Assay reagents (Bio-Rad, Hercules, CA). Caspase-3/7, caspase-8, and caspase-9 activities were measured using corresponding Caspase-Glo Assay kits (Promega, Madison, WI). Briefly, 50 μg of protein extracts of each tissue with equal volume were added to the Caspase-Glo reagents and caspase activity was measured at 2–3 h with a Victor 3 Multilabel Counter (PerkinElmer, Wellesley, MA). Measurements were performed in triplicate. Statistical analyses were performed using an unpaired *t* test for comparisons between the means of two independent groups. A *p* value was considered significant at <0.05.

Laser microdissection and genotyping of neural tissues. Embryos were fixed in either 10% formalin or Histochoice and embedded in paraffin as described above. Sections were cut at 6 μm thickness, collected onto 1 mm PEN (polyethylene naphthalate) membrane slides (Zeiss, Oberkochen, Germany), and stained with H&E or hematoxylin alone. Different neural structures were dissected from the sections using the Zeiss Microlaser System supplied with PALM (Bernried, Germany) RoboSoftware, version 1.2, and collected into a 0.5 ml cap. Samples were digested in TE buffer containing 1% Tween 20 and 2.5 mg/ml proteinase K at 65°C for 48 h. DNA was extracted using QIAamp DNA kit following the manufacturer's instructions. PCR primers used to detect the presence or absence of the modified *survivin* gene were Adv17, Adv25, and Adv 28 (Xing et al., 2004). When the *survivin* gene flanked by two *loxP* sites was present, a band of 560 bp was detected; when the *survivin* gene was absent, a band of 425 bp was detected.

Results

Nestin-cre expression during mouse development

To study the effects of *survivin* deficiency in neuronal progenitor cells *in vivo*, we crossed mice homozygous for the *survivin* gene flanked by two *loxP* sites (survivin$^{lox/lox}$) (Xing et al., 2004) with nestin-cre mice. Nestin is reported to be expressed in the mouse neural tube starting at E10 (Dahlstrand et al., 1995). At E15.5–P0, it is reportedly expressed in the cerebellum, the ventricular and subventricular telencephalon, and the radial glial cells (Dahlstrand et al., 1995). We characterized functional cre activity in our mice by crossing nestin-cre mice with cre reporter mice (B6; 129S-Gtrosa26$^{tm1sor)}$ (Soriano, 1999) (R26R) and assayed for β-galactosidase expression in the compound heterozygous mice at a variety of embryonic stages (E9.5–E14.5). On gross examination (whole mounts), X-gal staining was detected as early as

E10.5, with maximal staining at E11.5–E13.5 (supplemental Fig. 1*a*, available at www.jneurosci.org as supplemental material). Staining was strongly positive in the developing brain and spinal cord as well as in the eye and ear. Weak staining was also observed in a distal region within the limb buds (supplemental Fig. 1*a*, available at www.jneurosci.org as supplemental material). Microscopic examination of brain sections from E11.5 embryos revealed β-galactosidase expression within the developing brain, including the telencephalon, diencephalon, mesencephalon, metencephalon, myelencephalon, and the cranial nerve ganglia (supplemental Fig. 1*b*, available at www.jneurosci.org as supplemental material). Interestingly, a few areas within the developing brain were negative, for example, the optic recesses (entrance to the optic stalk) and the choroid invaginations (supplemental Fig. 1*b*, arrows; available at www.jneurosci.org as supplemental material).

Phenotype of nestin-cre;survivin$^{lox/lox}$ mice

Nestin-cre;survivin$^{lox/lox}$ mice survived to birth with an expected Mendelian ratio of 25%, indicating no embryonic lethality (supplemental Table 1, available at www.jneurosci.org as supplemental material). After assisted C-section delivery at E19.5, nestin-cre;survivin$^{lox/lox}$ neonates displayed normal responses to external stimuli, without overt motor defects. Vaginally and C-section delivered newborn mice became cyanotic and died a few minutes after delivery, most likely because of respiratory insufficiency as a result of abnormal brainstem function.

At birth, nestin-cre;survivin$^{lox/lox}$ mice had a normal body size when compared with their littermates. Nonetheless, the mutant mice were easily distinguishable by the abnormal morphology of their heads, characterized by a flattened cranium (Fig. 1*a*). Macroscopic examination of the brain revealed microencephaly, characterized by marked overall reduction in size of the brain and increased fragility of the remaining brain tissue (Fig. 1*b*). Microscopic evaluation of the brain showed marked diffuse cerebral and cerebellar hypoplasia with massive destruction of the neuronal progenitor cells. Compared with the cerebrum and cerebellum, the brainstem showed less destruction of neuronal tissue, but was overall reduced in size, accompanied by multifocal marked dysplasia of the remaining neuronal tissue (Fig. 1*c*). The spinal cord contained some areas of neuronal tissue destruction (apoptosis) within the dorsal and intermediolateral gray matter horns (see Fig. 9*a*). The peripheral nervous system, including the dorsal root and cranial ganglia showed no significant microscopic lesions (see Fig. 9*a*). The lungs of the mutant mice were minimally inflated (fetal atelectasis) suggesting inadequate air exchange; however, the remaining architecture of the lungs was intact by microscopic analysis. The heart, liver, diaphragm, and skeletal muscles appeared normal at the gross and microscopic levels at E16.5 and P0 (data not shown). The ability of these mice with such profound loss of brain structure to survive to term is reminiscent of mouse and human forms of anencephaly, such as the *MacMARCKS*-deficient mice that survive to term and to human examples of similar disorders (Chen et al., 1996; Walters et al., 1997).

To determine the rate of penetrance of the observed phenotype within the brain and spinal cord of the *survivin*-deficient mice, we performed gross and microscopic analyses of these regions in nestin-cre;survivin$^{lox/lox}$ embryos from E9.5 to P0 and correlated these with our genotype analyses. Supplemental Table 2 (available at www.jneurosci.org as supplemental material) shows the results of this analysis. A total of 44 embryos with homozygous loss of the *survivin* gene in a nestin-specific manner

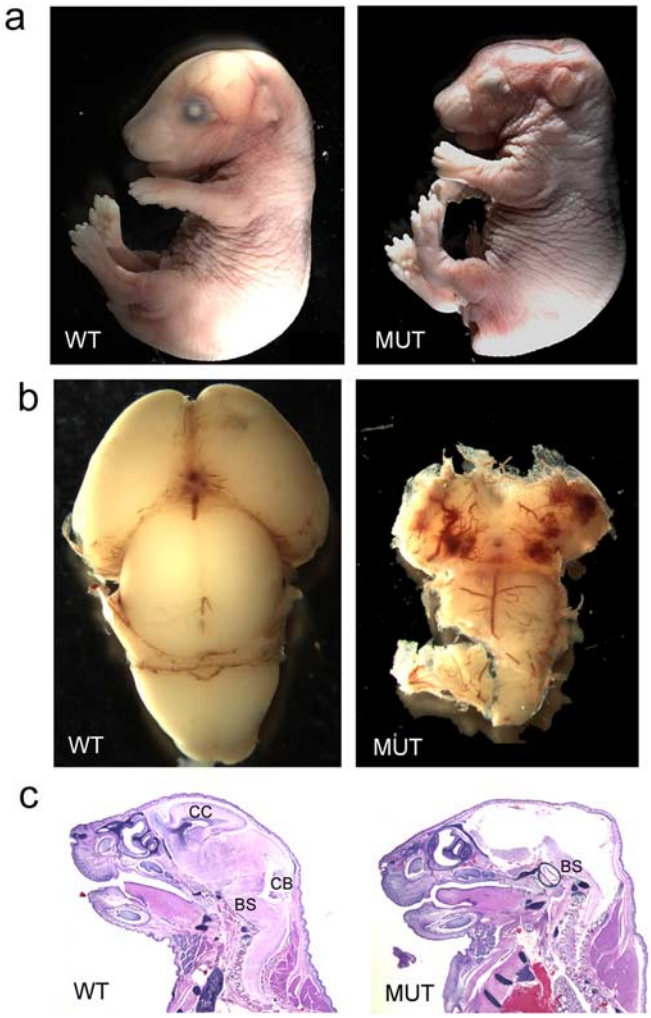

Figure 1. Brain malformation in nestin-cre;survivin$^{lox/lox}$ mice. ***a***, Whole mounts of E17.5 mice showing the abnormal head morphology of a mutant (MUT) compared with a wild-type (WT) littermate. ***b***, Dissected brain from mutant and wild-type embryos at E17.5. ***c***, Sagittal H&E-stained sections of mice at P0 showing the brain structures of survivin mutant and wild-type animals. The cerebral cortex (CC) and cerebellum (CB) were absent in the mutant brains. The brainstem (BS) was relatively preserved but smaller in the mutant than in the wild-type animals.

were examined. Forty-one of these embryos ranged in age from E10.5 to P0. Three embryos were obtained at E9.5, before the expression of nestin, as demonstrated in the nestin-cre;cre-reporter mice. All three E9.5 embryos had a normal neural tube when compared with their littermate controls. In contrast, 100% of E10.5 through P0 nestin-cre;survivin$^{lox/lox}$ embryos had abnormalities within the developing brains. This is consistent with a complete penetrance of the observed phenotype.

To address the potential mechanism by which some regions within the brain and spinal cord remained phenotypically unaffected by the loss of *survivin* gene expression, whereas others were dramatically disrupted, we isolated DNA from cells within these regions using laser capture microscopy. We then performed PCR amplification of the DNA using primers specific for the *survivin* gene (Xing et al., 2004). Results of these experiments are shown in supplemental Figure 2 (available at www.jneurosci.org as supplemental material). Cells isolated from the cerebral cortex, spinal cord, and brainstem from E12 or E14.5 nestin-cre;survivin$^{lox/lox}$ embryos showed complete deletion of the *survivin* gene in these sites, suggesting that additional survival factors must exist within

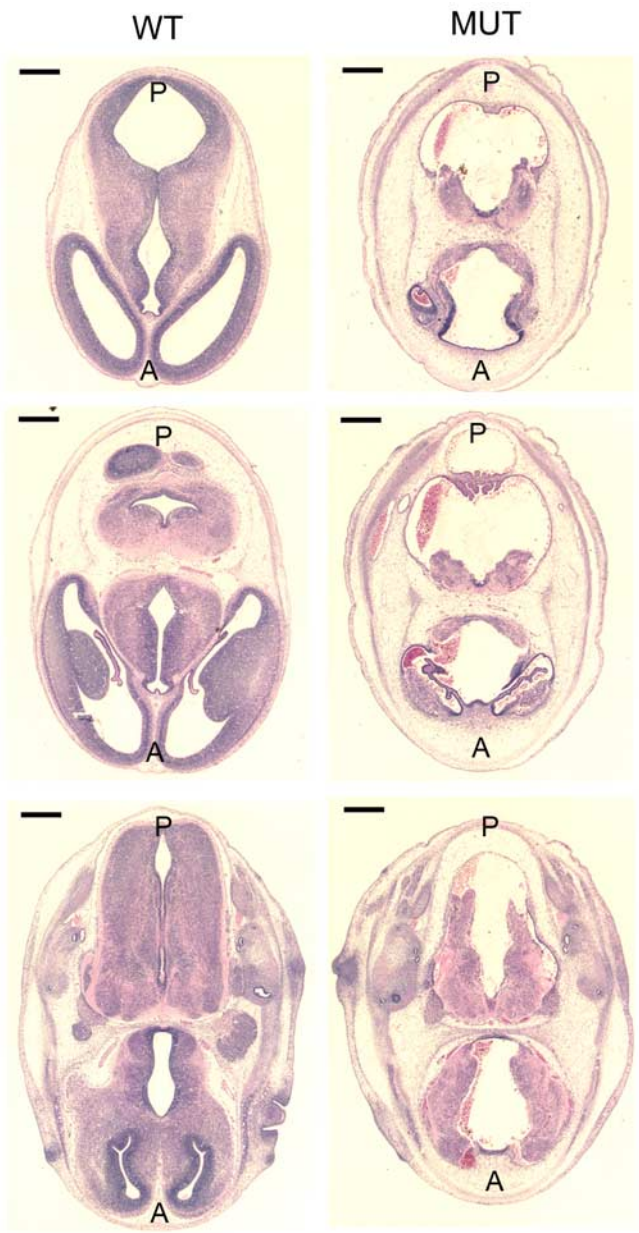

Figure 2. H&E-stained transverse sections at E13.5 showing multifocal destruction of brain architecture within the developing brain of the mutant embryos. Scale bars, 50 μm. WT, Wild type; MUT, mutant; A, anterior; P, posterior.

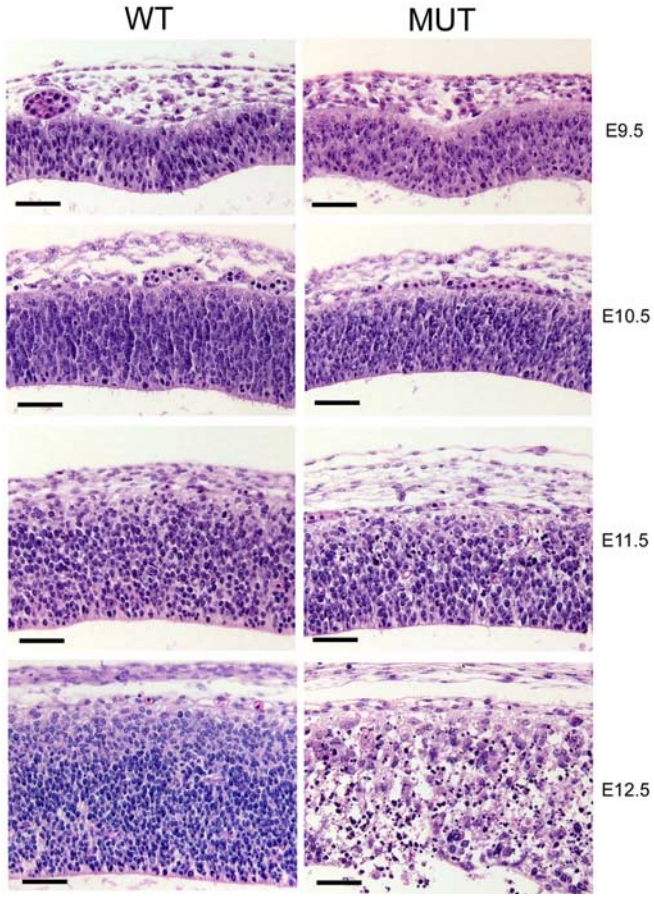

Figure 3. H&E-stained sections from pial (top) to ventricular (bottom) surface at E9.5–E12.5 showing progressive destruction of cortical architecture within the developing cerebral cortex of the mutant embryos. Scale bars, 500 μm. WT, Wild type; MUT, mutant.

the spinal cord to compensate for the loss of *survivin* there. In contrast, genotyping of cells isolated from the pons and retina showed that there was mosaicism of the *survivin* locus there, suggesting that the partial protection from cell death in these regions can be explained by the lack of complete loss of *survivin* in all cells in these regions.

Massive apoptosis in the brains of nestin-cre;survivin$^{lox/lox}$ mice

Cell death was readily detected by microscopic analysis of H&E-stained sections of brains from nestin-cre;survivin$^{lox/lox}$ embryos as early as E10.5, and loss of major brain structures was clearly evident at E12.5 and E13.5 (Figs. 2 and 3). At E10.5–E12.5, a progressive increase in the number of TUNEL-positive cells was observed in the developing cerebral cortex (Fig. 4). The mean TUNEL counts for the knock-out embryos (mean ± SD, 32.3 ± 11.5) were significantly greater than the counts for the wild-type group (mean ± SD, 1.7 ± 1.28; $p = 0.01$).

To assess the effects of *survivin* deletion on proliferation in the developing cerebral cortex, we performed several different assays. These included BrdU, PCNA, and phosphohistone H3 staining of dissected sections within the brain and spinal cord. BrdU staining for proliferating cell populations within the brain revealed only slightly fewer BrdU-positive cells at E11.5 in the mutant, compared with wild-type embryos. However, at E12.5–E13.5, there were significantly fewer BrdU-positive cells (data not shown), likely reflecting the increase in cell death occurring in the developing cortex and cerebellum of the nestin-cre;survivin$^{lox/lox}$ embryos. PCNA, a protein that plays a critical role in DNA replication, is expressed during all phases of cell division. Although high levels of PCNA expression were observed throughout the layers of the developing cortex in the nestin-cre;survivin$^{lox/lox}$ embryos, small differences in expression (average 10%) were observed in wild-type versus mutant embryos at E9.5–E11.5 that were not statistically significant (Fig. 5). Larger differences were observed in the mutant embryos at E12.5, again paralleling an increase in the number of apoptotic cells. PHH3 is a specific marker for mitosis and is rapidly degraded on entry into G_1 (Shibata and Ajiro, 1993). Antibodies to the phosphorylated form of histone H3 are used to increase the sensitivity of identifying mitosis-specific cell cycle events. PHH3 staining of embryonic brain sections from E9.5 to E12.5 revealed small, but significant differ-

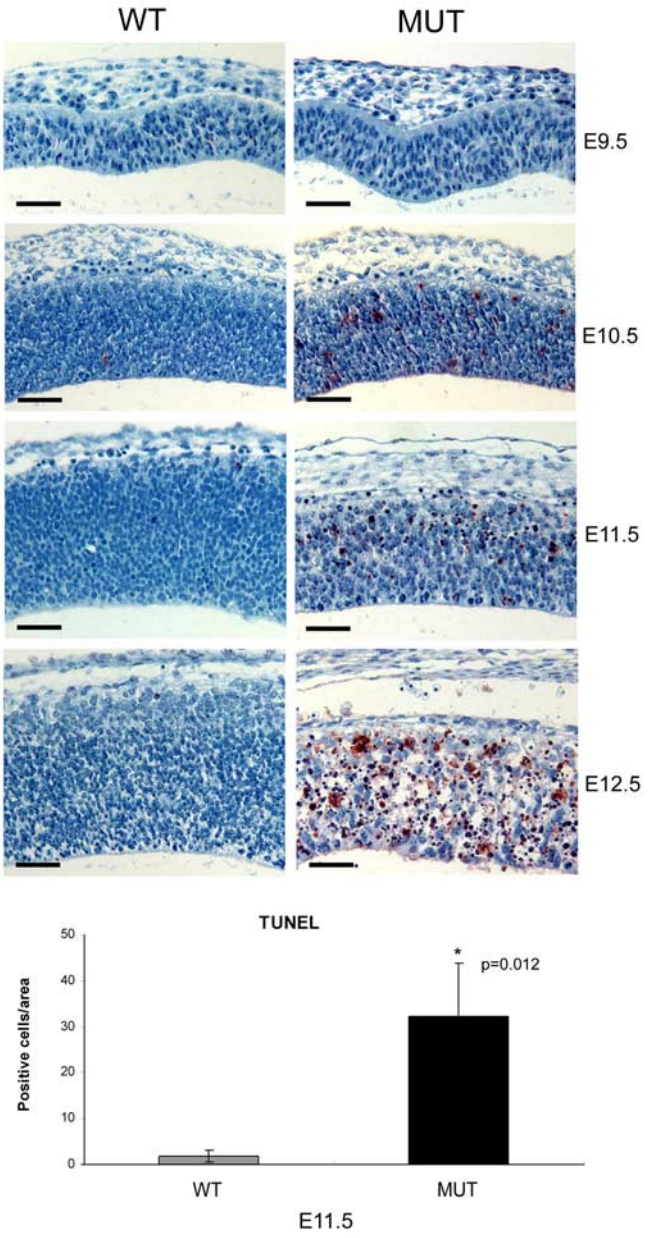

Figure 4. Top, TUNEL assays showing progressive increases in apoptotic cell death within the cerebral cortex of the mutant embryos, starting at E10.5. Scale bars, 500 μm. Bottom, A histogram depicts the average number of TUNEL-positive cells per area for the wild-type (WT) and mutant (MUT) animals. *Statistically significant difference ($p = 0.01$). Error bars indicate SD.

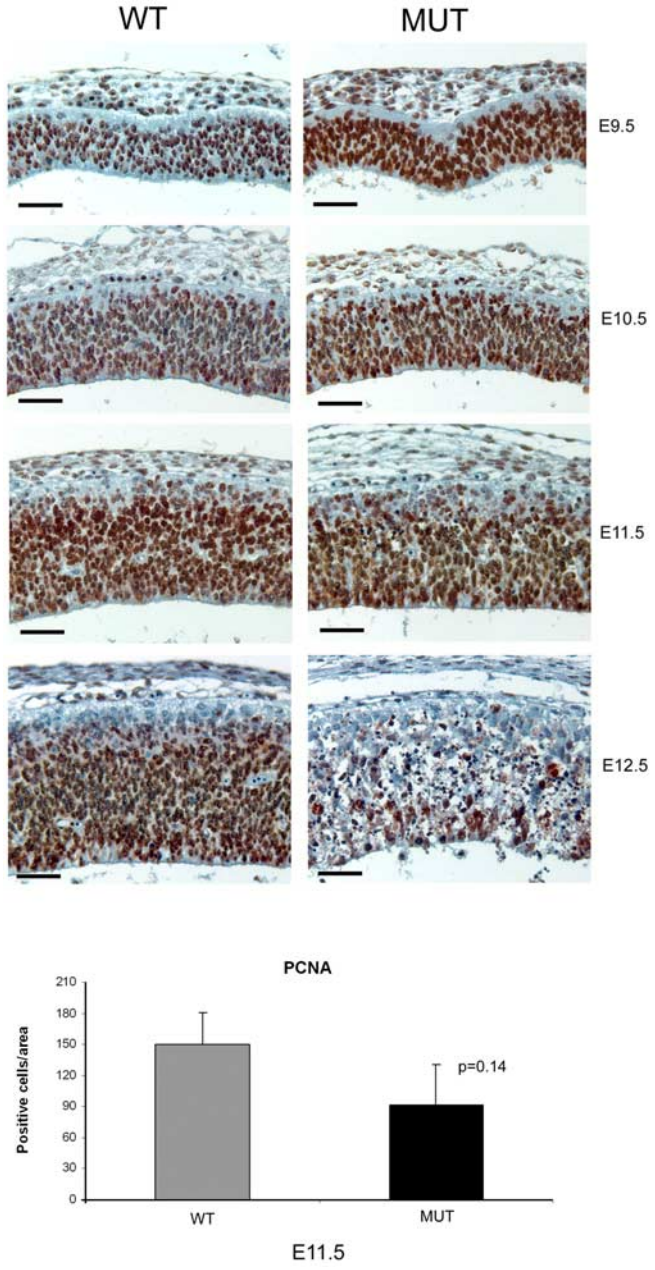

Figure 5. Top, PCNA staining showing small differences in the number of proliferating cells within the cerebral cortex between mutant and wild-type animals. Scale bars, 500 μm. Bottom, A histogram depicts the average number of PCNA-positive cells per area for the wild-type (WT) and mutant (MUT) animals. No statistical differences were observed. Error bars indicate SD.

ences in the levels of expression throughout these time points (Fig. 6). The mean PHH3 counts for wild-type embryos (mean ± SD, 9.1 ± 1.62) were significantly greater than the counts for the knock-out group (mean ± SD, 3.8 ± 0.71; $p = 0.02$). A 10–20% decrease in expression of PHH3 was observed as early as E10.5, suggesting that survivin may have some effect on the ability of the developing periventricular cells to enter or complete mitosis. This effect, however, does not appear to be the primary cause for the massive disruption in brain development observed in these animals, based on the significantly higher increased levels of apoptosis in the mutant animals. Interestingly, when comparing the pattern of cells undergoing apoptosis to the pattern of cells in the various phases of cell division, neural cells undergoing cell death were not confined to the G_2/M phase of the cell cycle, but occurred throughout all phases of cell division.

To understand the cellular events after *survivin* disruption, we measured activated caspase activity by IHC using antibodies to the active form of caspase-3/7 and by a luciferase-based chemical assay for caspase-3/7, -8, and -9, using freshly isolated brain tissue. Significant increases in activated caspase-3 expression were observed from E10.5 through E12.5 in the mutant brains, as shown by IHC staining (Fig. 7). The mean caspase-3 counts for the knock-out embryos (mean ± SD, 28.75 ± 2.37) were significantly greater than the counts for the wild-type group (mean ± SD, 0.5 ± 0.76; $p < 0.001$). At E10.5 and E12, caspase-3/7 activity was 5 and 10 times higher in the mutant brains than in the wild-type brains, respectively (Fig. 8). In addition, caspase-9 activity was similarly increased in the affected brains. The relative increase in caspase-3/7 and caspase-9 activities was much greater

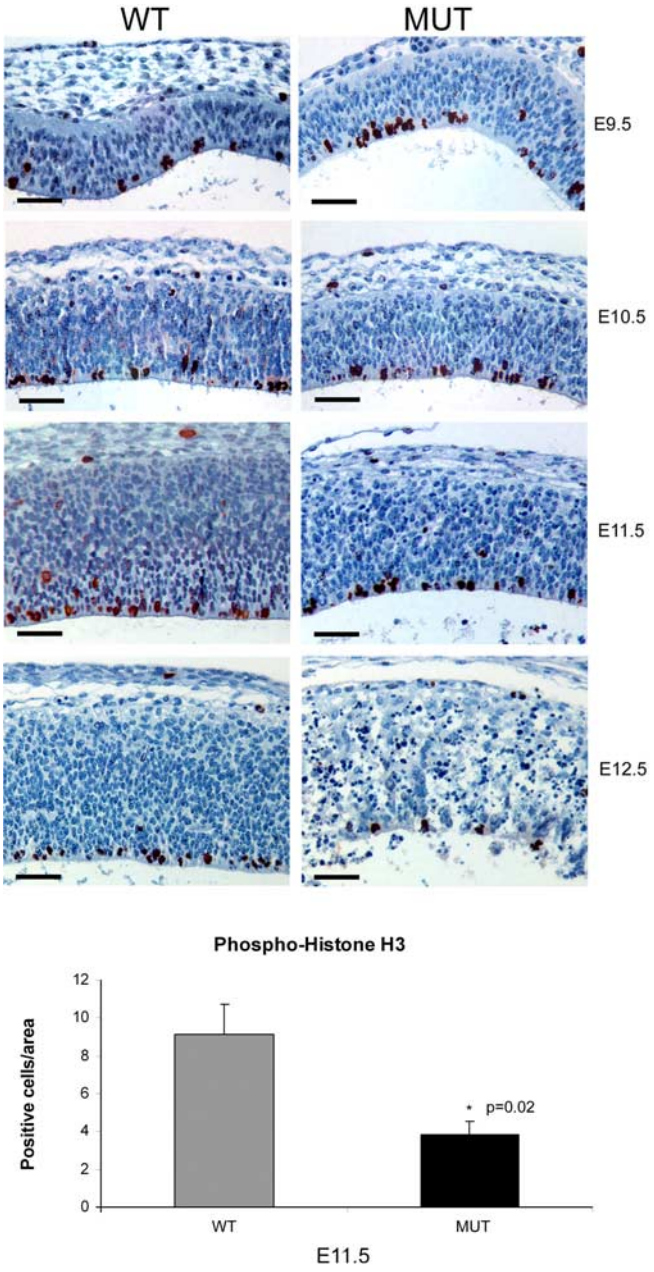

Figure 6. Top, PHH3 expression showing a moderate decrease in the number of cells in G_2/M in the cerebral cortex of the mutant embryos. Scale bars, 500 μm. Bottom, A histogram depicts the average number of PHH3-positive cells per area for the wild-type (WT) and mutant (MUT) animals. *Statistically significant difference ($p = 0.02$). Error bars indicate SD.

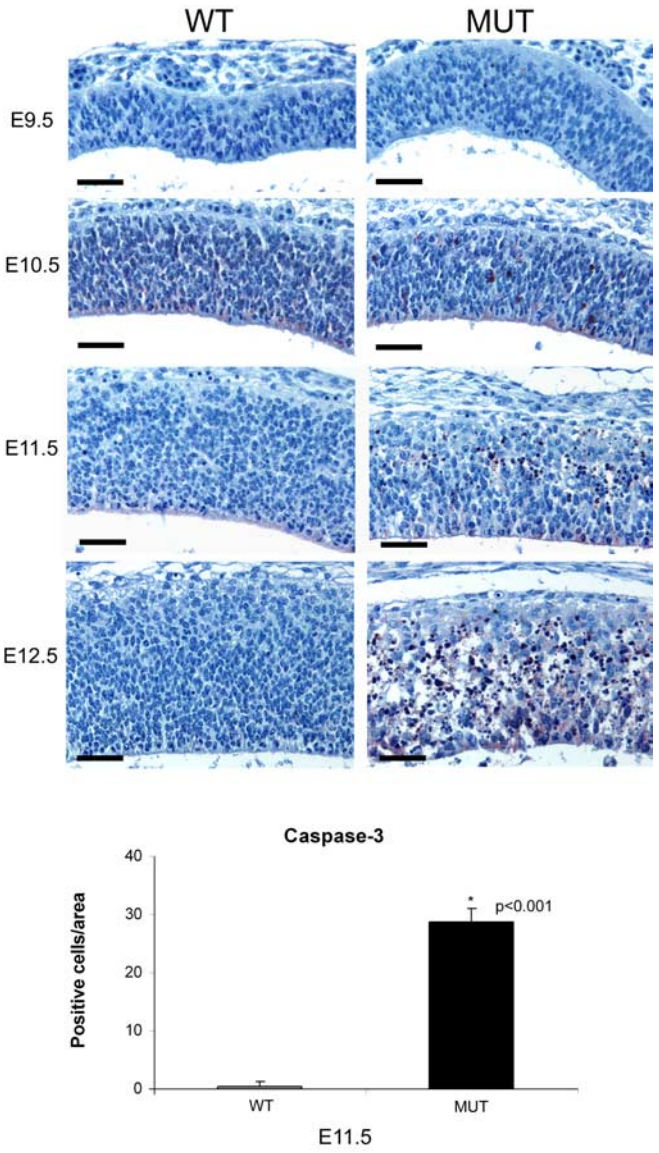

Figure 7. Activated caspase expression in the developing cortex of nestin-cre;survivin$^{lox/lox}$ embryos. Top, Sections from the cerebral cortex from E9.5 through E12.5 were stained with activated caspase-3 antibodies. A progressive increase in expression of activated caspase-3 is seen, starting at E10.5. Scale bars, 50 μm. Bottom, A histogram depicts the average number of caspase-3-positive cells per area for the wild-type (WT) and mutant (MUT) animals. *Statistically significant difference ($p < 0.001$). Error bars indicate SD.

than that observed with caspase-8 (Fig. 8). This is consistent with a more prominent role of survivin in caspase-9 and caspase-3 inhibition (Tyagi et al., 2003; Chandele et al., 2004; Pennati et al., 2004).

We also evaluated the expression of the proapoptotic bcl-2 family member bax by immunohistochemistry. At E12.5 and E13.5, bax was highly expressed in both the mutant and littermate control brains within neural cells lining the ventricles, choroid plexus, retina, cranial ganglia, and the ventral horn of the spinal cord (data not shown). There were no differences in the expression of bax between *survivin*-deficient and control brains, indicating *survivin* disruption does not alter the levels of bax. This result is consistent with the proposed antiapoptotic mechanism of survivin, downstream of the bcl-2 family of apoptotic regulators.

Neuronal disorganization within the brainstem and spinal cord

The neuronal tissue within the brainstem and spinal cord appeared multifocally disrupted and disorganized. These abnormalities most likely resulted in an inability of the newborn mice to coordinate basic respiratory functions, resulting in their rapid death after birth. The dorsal and intermediolateral horns within the gray matter of the spinal cord were most affected by the loss of *survivin* with an increase in apoptotic cells noted on H&E (Fig. 9*a*). TUNEL staining within the spinal cord showed a moderate increase in apoptotic cells in this region (data not shown). In addition, the spinal cord of conditional mutant embryos contained multiple abnormally shaped giant cells characterized by

karyomegaly and cytomegaly with extensive cytoplasmic processes (Fig. 9*b*). Staining with markers of neuronal and astrocytic differentiation showed that the giant cells were GFAP positive, suggesting that they were of an astrocytic lineage. Myelinated fibers were also dysplastic, forming tangled bundles in the brain and spinal cord of the mutant animals, as demonstrated by Luxol fast blue staining (data not shown).

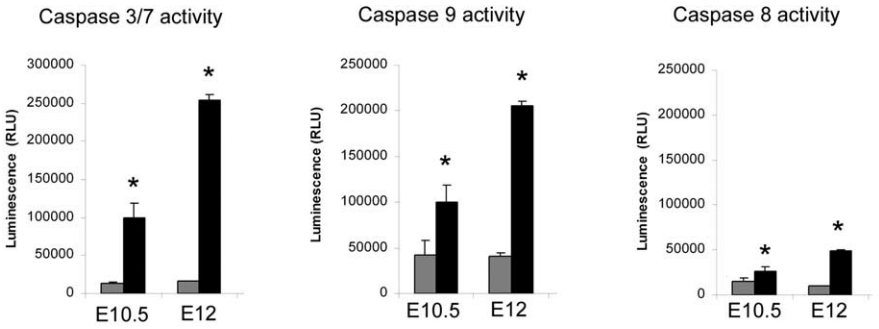

Figure 8. Functional caspase activity in the developing cortex of nestin-cre;survivin$^{lox/lox}$ embryos. Developing cortical tissues were evaluated for functional caspase-3/7, -8, and -9 activities at E10.5 and E12 using a luciferase-based chemical assay system (Caspase-Glo; Promega). Relative luciferase units (RLU) are proportional to functional caspase activity, as indicated by the manufacturer. Gray columns represent the samples from wild-type animals, and black columns represent the samples from the mutants. Three animals in each group were used ($n = 3$). *Statistical significance ($p < 0.001$ for all). Error bars indicate SD.

Retinal defects in nestin-cre;survivin$^{lox/lox}$ mice

Because the retina is an extension of the CNS derived from neural ectoderm and because it expresses nestin-cre, we also investigated the role of survivin in retinal development. Nestin-cre; survivin$^{lox/lox}$ mice displayed increased apoptotic cell death within the retina, corresponding to the loss of *survivin* in some of the cells within this organ (supplemental Fig. 2, available at www.jneurosci.org as supplemental material). At E17.5, the retina showed significantly elevated numbers of apoptotic cells in both the external and internal cell layers, when compared with littermate controls (Fig. 10). No changes were seen in the number of BrdU-positive cells in the retina at this stage (data not shown).

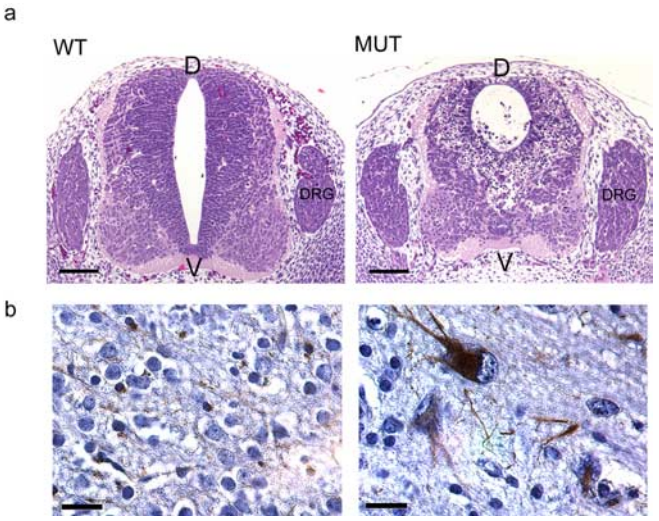

Figure 9. Effects of a lack of survivin within the developing spinal cord. ***a***, Sections of the developing spinal cord from E12 embryos were stained with H&E. Neuronal disorganization and cell death is observed. DRG, Dorsal root ganglia; D, dorsal; V, ventral. Scale bars, 100 μm. ***b***, Sections of spinal cords from mutant (MUT) mice and wild-type (WT) littermates at P0 were stained with GFAP. Giant cells observed in the mutant animals expressed GFAP, suggesting they are of an astrocytic lineage. Scale bars, 25 μm.

Discussion

In this work, we show that *survivin* plays a critical role in protecting developing neuronal cells within the CNS from deregulated apoptosis. When *survivin* expression is disrupted in the developing brain in a nestin-specific manner, embryos survive until birth, but most of the brain is hypoplastic. On a cellular level, this phenotype occurs as a result of massive neuronal apoptosis involving the developing cerebral cortex, cerebellum, gray matter of the spinal cord, and retina. Thus, genetic disruption of *survivin* may be useful as a model to study human developmental disorders of neuronal cell death as well as for neurodegenerative disease.

The exact molecular mechanism(s) by which survivin inhibits apoptosis is unclear. Its crystallographic structure is notable for the absence of a caspase-binding domain [CARD (caspase recruitment domain)], unlike other IAP proteins, such as c-IAP-1, and c-IAP-2 (Salvesen and Duckett, 2002). Therefore, proposals of its direct binding ability to caspase proteins would imply alternative, as-yet-undefined molecular interactions. It has been shown to bind Smac (second mitochondria-derived activator of caspase)/DIABLO (direct IAP binding protein with low pI) and AIF (apoptosis-inducing factor) *in vitro*, the binding of which has been reported to be required for their antiapoptotic activity (Shankar et al., 2001; Song et al., 2003). Most recently, it has been proposed that a subcellular mitochondrial pool of survivin is predominantly responsible for its antiapoptotic activity through inhibition of caspase-9 activation and the formation of the apoptosome complex (Dohi et al., 2004). Our results showing an increase in the activity of caspase-3 and caspase-9 in the *survivin*-null brains support the role of survivin in inhibiting caspase-3 and caspase-9 activity *in vivo*, through either indirect or direct mechanisms.

In addition to its function as an antiapoptotic protein, survivin has a role in mitosis as a CPP (Skoufias et al., 2000; Wheatley et al., 2001). In this capacity, it recruits aurora B kinase to the CPP complex and ensures the proper alignment of chromosomes before cell division (Chen et al., 2003). In their original targeted genetic approach, Uren et al. (2000) showed that homozygous deletion of *survivin* resulted in embryonic lethality between E4 and E6. The majority of *survivin*$^{-/-}$ embryos had grossly enlarged nuclei with abnormal morphology. As this phenotype progressed, the cells ceased to complete mitosis, with the decreasing number of normal cells replaced by a small number of giant cells with large and morphologically unusual nuclei. In our work, we do observe some enlarged GFAP-positive nuclei within the spinal cord at P0. We also see evidence of a diminishing effect on the population of developing neuronal cells within the cerebral cortex in the mitotic phase of cell division. However, the predominant response to a lack of neuronal cell survivin is a major increase in neuronal apoptosis. These effects on apoptotic pathways are likely most responsible for the observed profound alterations in brain development in the nestin-cre;*survivin*$^{-/-}$ embryos. The findings moreover exemplify the various roles that the *survivin* gene family plays in different cell types *in vivo*.

Apoptosis has been recognized to be an essential process dur-

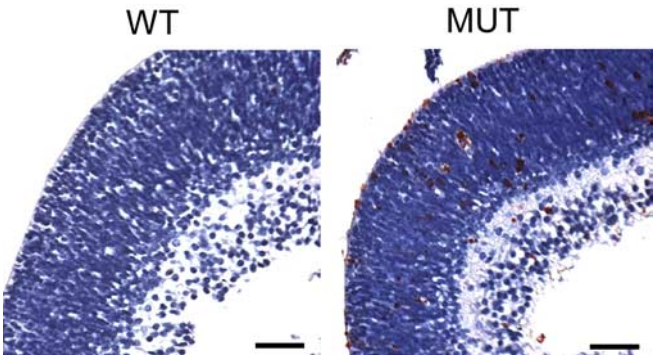

Figure 10. Retinal defects in nestin-cre;survivin $^{lox/lox}$ mice. TUNEL assays show increased apoptosis in the retina at E17.5 in the nestin-cre;survivin $^{lox/lox}$ embryos. Apoptotic nuclei appear dark red. Scale bars, 50 μm. WT, Wild type; MUT, mutant.

ing normal neuronal development in which it appears to control the final number of neurons in the CNS and PNS. Two distinct periods of cell death are observed: a trophic factor-dependent cell death involving postmitotic neurons and a cell death involving progenitor neurons that is independent from synaptogenesis (for review, see Lossi and Merighi, 2003). Mouse genetic studies have provided evidence of the involvement of multiple genes in regulating progenitor neuronal cell death *in vivo*. These include the classic mitochondrial cell death genes within the *bcl-2* family, downstream cell death effector *caspases*, genes involved in maintaining DNA stability, as well as the tumor suppressor gene *Pten* (Motoyama et al., 1995; Deckwerth et al., 1996; Kuida et al., 1996; Hakem et al., 1998; Groszer et al., 2001). Disruption of the anti-apoptotic *bcl-x_L* gene leads to death at E13, with embryos showing massive apoptosis in the developing spinal cord, brainstem, and dorsal root ganglia (Motoyama et al., 1995). Mice deficient for both *bcl-x_L* and the proapoptotic gene *bax*, show attenuated neuronal cell death within the CNS, suggesting that both of these genes are important for early neuronal survival (Deckwerth et al., 1996; White et al., 1998). *Caspase-3* (Kuida et al., 1996)- and *caspase-9* (Hakem et al., 1998)-deficient mice have defects within the CNS that include neuronal hyperplasia of the cortex, cerebellum, striatum, hippocampus, and retina, and neuronal disorganization. Some proteins involved in recognizing DNA strand breaks (DSBs), known as NHEJ (nonhomologous end joining) proteins that include XRCC4 (X-ray repair cross-complementing protein 4) and ligase IV, are critical for the survival of newly generated postmitotic neurons, suggesting that DSBs occur at high rates in early postmitotic neurons (Gao et al., 1998). The results from our study, showing that the levels of bax are unchanged in the *survivin*-deficient versus the wild-type brains, but that caspase-3 and caspase-9 are activated at much higher levels in the *survivin*-deficient brains, are consistent with the role of survivin downstream of the bcl-2 proteins, in inhibiting caspase-9 and the effector caspases 3 and 7 in progenitor neurons. Our study is the first *in vivo* study that supports this view. Future studies examining the phenotype of mice deficient for both *survivin* and *caspase-3* or *-9* should be able to functionally address these findings.

Estimates of the magnitude of naturally occurring cell death of neural progenitor cells are conflicting and appear to vary with the method of detection. Some studies, using assays that include TUNEL, activated caspase-3, and annexin V as detection methods, report a death rate of $<$2% in the developing cortex at mid-neurogenesis (Vermes et al., 1995; Thomaidou et al., 1997; Roth et al., 2000). In contrast, other studies have shown up to 70% of newly made neurons within the cerebral cortex undergo apoptosis at E14 using a variation of TUNEL method called ISEL+ (*in situ* end-labeling plus) (Blaschke et al., 1996). Recent data from experiments using labeled telencephalic clones in *Dct-LaacZ* mosaic embryos to study the early stages of neurogenesis *in vivo* also supported the possibility of substantial increases in cell death rates of progenitor neurons during E13–E14 (Wilkie et al., 2004). Regardless, our data show that protection against apoptosis during neurogenesis is crucial for normal development of the brain and PNS, and that survivin plays a central role in this process.

Overall, our studies clearly demonstrate that survivin is an essential survival factor *in vivo* during the development of the mammalian nervous system. We hypothesize that survivin expression is critical to maintain cell survival in cells that are continuously proliferating during CNS development. The central role of survivin in preventing cell death of developing neuronal precursors as well as of postmitotic neurons should be explored further, particularly in the context of disease models of neural development and neurodegeneration.

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