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Strain-Dependent Neurodevelopmental Abnormalities in Caspase-3-Deficient Mice

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Abstract. Targeted gene disruptions have revealed significant roles for caspase family members in the regulation of neuronal programmed cell death. Both caspase-3- and caspase-9-deficient mice exhibit a variably severe neurodevelopmental phenotype that may include marked ventricular zone expansion, exencephaly, and ectopic neuronal structures. Our previous studies of caspase-3- and caspase-9-deficient mice were performed using mice on mixed genetic backgrounds, raising the possibility that strain-specific genetic factors influence the effects of caspase deficiency on nervous system development. To directly test this hypothesis, we backcrossed the *caspase-3* mutation for 7–10 generations onto pure C57BL/6J and 129X1/SvJ genetic backgrounds. Caspase-3-deficient 129X1/SvJ mice were uniformly and severely affected. These mice died during the perinatal period and exhibited marked neural precursor cell expansion and exencephaly. In contrast, caspase-3-deficient C57BL/6J mice reached adulthood, were fertile and showed minimal brain pathology. Intercrosses of C57BL/6J and 129X1/SvJ mutants revealed that the vast majority of *caspase-3*$^{-/-}$ F1 mice displayed the severe 129X1/SvJ-''like'' phenotype. These findings are consistent with an incompletely penetrant strain-dependent genetic modifier (or modifiers) that alters the neurodevelopmental consequences of caspase-3 deficiency. Since caspase-9- and Apaf-1-deficient mice also display variably severe developmental neuropathology, this strain-dependent modifier(s) may be involved in the activation of a caspase-independent death pathway; alternatively, strain-dependent compensatory caspase activation and/or its inhibition may influence the severity of the caspase-3-deficient neuronal phenotype.

Key Words: Apaf-1; Apoptosis; Bcl-2; Caspase-9; Programmed cell death; Telencephalon.

INTRODUCTION

Programmed cell death plays an important role in nervous system development (1). In *Caenorhabditis elegans*, programmed cell death is regulated by EGL-1, CED-9, CED-4, and CED-3 (2). Homologues of these molecules exist in mammals and consist of members of the BH3 domain-only, Bcl-2, Apaf-1, and caspase families, respectively. Caspases are cysteine-containing, aspartate-specific proteases that are expressed as inactive proenzymes and are cleaved into 2 active subunits in response to apoptotic stimuli (3). Caspases can be divided into several subgroups based on structure, function, and/or position in the apoptotic cascade. Targeted gene disruptions of many caspases have been reported, yet only mutations in *caspase-9* and *caspase-3* have resulted in severe developmental brain abnormalities (4).

The majority of caspase-3- and caspase-9-deficient mice died at or shortly after birth and displayed similar gross neurodevelopmental lesions as Apaf-1-deficient mice (5–7). Affected embryos showed decreased programmed cell death in brain regions involved in brain morphogenesis, such as the lamina terminalis and tectal roofplate, and exhibited marked hyperplasia of the embryonic ventricular zone. Severely affected mice showed exencephaly, ectopic and duplicated neuronal structures and supernumerary cells that often obliterated the intraventricular space.

The targeted mutation of *caspase-3* was originally accomplished in 129/Sv ES cells and chimeric mice were then generated in C57BL/6J recipients. Following germline transmission of the mutation, homozygous *caspase-3* mutants were generated by brother-sister matings. Although the vast majority of caspase-3-deficient mice died in the perinatal period and exhibited severe developmental brain abnormalities, a small percentage survived into adulthood. Similarly, a subset of caspase-9- and Apaf-1-deficient mice were also viable and showed more subtle brain abnormalities. These observations suggested that mouse strain-dependent genetic factors modified the effect of caspase-3 on brain development. To definitively test this hypothesis, we systematically backcrossed the *caspase-3* mutation onto the 129X1/SvJ and C57BL/6J genetic backgrounds. The results from our investigation prove that strain-specific genetic modifiers influence the caspase-3-deficient neuronal phenotype.

MATERIALS AND METHODS

Generation of Caspase-3 Mutant Mice

The generation and characterization of *caspase-3*$^{-/-}$ mice on a mixed genetic background has been described previously (5). To generate pure C57BL/6J and 129X1/SvJ *caspase-3*$^{-/-}$ mice, genetically mixed *caspase-3*$^{+/-}$ mice were systematically backcrossed with wild-type C57BL/6J and 129X1/SvJ mice. The effects of caspase-3 deficiency on enriched C57BL/6J and

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129X1/SvJ genetic backgrounds were assessed after 7–10 generations of backcrosses.

Histology and Immunohistochemistry

Pregnant mice were anesthetized with methoxyflurane and killed between gestational day 12.5 and 18 by cervical dislocation. Embryos (E12.5–E18) were removed and tail and limb samples were taken for DNA extractions and PCR analyses. Neonatal and adult brains were collected from mice after methoxyflurane anesthesia, cervical dislocation, and decapitation. Whole embryos or brains were placed in Bouin's fixative overnight at 4°C followed by 3 washes in 70% ethanol. Tissue was dehydrated, paraffin-embedded, and 4-μm-thick sagittal sections were cut. Sections were deparaffinized and hematoxylin and eosin (H&E)-stained as described previously (8).

For immunohistochemistry, deparaffinized sections were incubated overnight at 4°C with CM-1, an affinity-purified rabbit polyclonal antiserum, which recognizes the p18 subunit of activated caspase-3 (9), diluted 1:40,000 in PBS-blocking buffer (PBS-BB; PBS with 0.1% BSA, 0.3% Triton X-100 and 0.2% nonfat powdered dry milk). Following washes with PBS, sections were incubated with a donkey anti-rabbit horseradish peroxidase-conjugated secondary antiserum (Jackson Immunoresearch, West Grove, PA), diluted 1:1,000 in PBS-BB, for 1 hour (h) at room temperature. Immunostaining was detected using tyramide signal amplification (Perkin-Elmer Life Science Products, Boston, MA). Tissue was counterstained with bisbenzimide (0.2 μg/mL; Hoechst 33,258; Sigma, St. Louis, MO) and examined with a Zeiss-Axioskop microscope equipped with epifluorescence.

Primary Telencephalic Cell Preparations

Primary cell cultures were prepared as described previously (10, 11). Briefly, embryos were removed between gestational days 12 and 13, telencephalic vesicles were isolated, and cells were dissociated for 15 min at 37°C in HBSS (Gibco, Grand Island, NY) containing 0.01% trypsin with 0.004% EDTA, 0.02 mg/ml DNase I, and 0.1% BSA (all purchased from Sigma). Trypsinization was stopped by adding an equal volume of HBSS containing 10% fetal calf serum (FCS). Cells were further dissociated by 3 rounds of trituration with a fire-polished Pasteur pipette and washed once with HBSS. A small sample was stained with Trypan Blue and counted. Approximately 2 million viable cells per embryo were collected. For induction of apoptosis, freshly dissociated cells at a concentration of 1.0×10^6 cells/ml were treated with 100 μM cytosine arabinoside (AraC; Sigma) or γ-irradiated (10 Gy) and incubated at 37°C in humidified 5% CO_2/95% air atmosphere. Six h later, cells were consecutively labeled with 75 nM MitoTracker Red CMXRos (Molecular Probes, Eugene, OR) and 2.5 nM SYTOX Green (Molecular Probes) and analyzed by flow cytometry as described previously (12).

RESULTS

Caspase-3 Deficiency Produces Strain-Specific Perinatal Lethality

The *caspase-3* mutation was bred for 7–10 generations onto the 129X1/SvJ and C57BL/6J genetic backgrounds, at which point greater than 98% genetic ''purity'' was obtained. We first examined the effect of genetic background on the survival of *caspase-3*$^{-/-}$ mice by crossing genetically pure *caspase-3*$^{+/-}$ mice and examining the genotype of surviving animals at weaning (Table 1).

TABLE 1
Distribution of Caspase-3 Genotypes in Surviving Mice Generated from *Caspase-3*$^{+/-}$ × *Caspase-3*$^{+/-}$ Matings

	Percentage (Actual Number)		
	+/+	+/−	−/−
Expected	25	50	25
C57BL/6J	27 (21)	56 (43)	17 (13)
129X1/SvJ	35 (28)*	65 (51)*	0 (0)*

The frequency of *caspase-3*$^{+/+}$, and +/−, and −/− mice was determined by PCR analysis of tail DNA extracts prepared from weanling mice. To determine whether the distribution of generated genotypes followed the expected Mendelian distribution, χ^2 analysis of contingency tables was used.
* $p < 0.01$ versus expected.

We found no surviving *caspase-3*$^{-/-}$ 129X1/SvJ mice at weaning; in contrast, *caspase-3*$^{-/-}$ C57BL/6J mice were observed at the expected Mendelian frequency (Table 1). The vast majority of caspase-3-deficient C57BL/6J adult mice appeared grossly normal, had no obvious neurological abnormalities, and were fertile. Three of 40 adult caspase-3-deficient C57BL/6J mice showed gross and microscopic evidence of hydrocephalus. The etiology of the hydrocephalus in these few mice was not obvious on histopathological examination. In total, these observations indicate that caspase-3 deficiency has surprisingly little effect on the C57BL/6J genetic background and that the previously reported variable perinatal lethality of caspase-3-deficient mice was likely due to strain-dependent genetic modifiers present in the genetically mixed animals analyzed.

Strain-Specific Developmental Brain Pathology in Caspase-3-Deficient Mice

To determine if the failure to observe caspase-3-deficient weanling mice on the 129X1/SvJ genetic background was due to developmental brain abnormalities in such mice, we interbred *caspase-3*$^{+/-}$ 129X1/SvJ mice and assessed the genotypes and brain development of the resultant embryos between gestational days 12 and 16. Forty-three embryos were genotyped of which 9 (21%) were *caspase-3*$^{-/-}$, indicating that *caspase-3*$^{-/-}$ 129X1/SvJ embryos were generated at the expected Mendelian frequency for +/− crosses (i.e. 25%). Seven of the 9 *caspase-3*$^{-/-}$ embryos had severe gross brain and craniofacial abnormalities at embryo harvest (Table 2). All 9 *caspase-3*$^{-/-}$ embryos had neural precursor cell expansion and significant neurodevelopmental abnormalities on microscopic examination (Fig. 1A). These abnormalities

TABLE 2
Frequency of Developmental Brain Abnormalities in Caspase-3$^{-/-}$ Embryos

Strain	Gross Abnormalities	Microscopic Abnormalities
C57BL/6J	4% (1/24)*	4% (1/24)*
129X1/SvJ	78% (7/9)	100% (9/9)
F1	33% (3/9)	89% (8/9)

Gross craniofacial abnormalities were assessed at embryo harvest, prior to knowledge of embryonic genotypes. Bouin's fixed sagittal sections from paraffin-embedded E12.5–E16.5 mice were evaluated by an observer blinded to the embryos genetic background and *caspase-3* genotype.

* Note that the single abnormal C57BL/6J *caspase-3*$^{-/-}$ embryo was observed in a litter after 7 generations of backcrosses and abnormal embryos have not been observed in later generations.

were identical to those previously reported for severely affected caspase-3-, caspase-9-, and Apaf-1-deficient embryos. In contrast, analysis of 24 *caspase-3*$^{-/-}$ embryos from the seventh to tenth generation of C57BL/6J backcrosses revealed a single abnormal embryo in the seventh generation (Fig. 1B; Table 2). Activated caspase-3-like immunoreactivity was readily detected in the nervous systems of wild-type and heterozygote embryos from both strains. Caspase-3-deficient embryos of both genetic backgrounds exhibited only rare activated caspase-3-like immunoreactive neurons (data not shown). These rare cells probably express caspase-7, an effector caspase with which the CM1 antiserum is known to cross-react (9). These studies indicate that caspase-3 deficiency produces marked developmental brain pathology in 129X1/SvJ, but not C57BL/6J, mice.

Caspase-3$^{-/-}$ F1 Mice Exhibit a Variably Severe Neurodevelopmental Phenotype

If there is a completely penetrant dominant modifier gene that affects the caspase-3-deficient phenotype, caspase-3-deficient F1 mice (generated by crossing pure *caspase-3*$^{-/-}$ C57BL/6J and *caspase-3*$^{+/-}$ 129X1/SvJ mice) should be identical to *caspase-3*$^{-/-}$ mice of the dominant parental genotype. We initially examined *caspase-3*$^{-/-}$ F1 embryos (gestational days 13–16) for gross and microscopic developmental brain abnormalities and compared their frequency with that observed for *caspase-3*$^{-/-}$ 129X1/SvJ and C57BL/6J embryos (Table 2).

Caspase-3-deficient F1 mice displayed an intermediate frequency of the abnormal phenotype between *caspase-3*$^{-/-}$ 129X1/SvJ and C57BL/6J mice. Gross craniofacial abnormalities were observed in 33% and microscopic neurodevelopmental abnormalities in 89% of *caspase-3*$^{-/-}$ F1 embryos (Table 2). This result indicates that the modifying effect of mouse strain genotype on the *caspase-3* mutant phenotype is not due to a completely penetrant

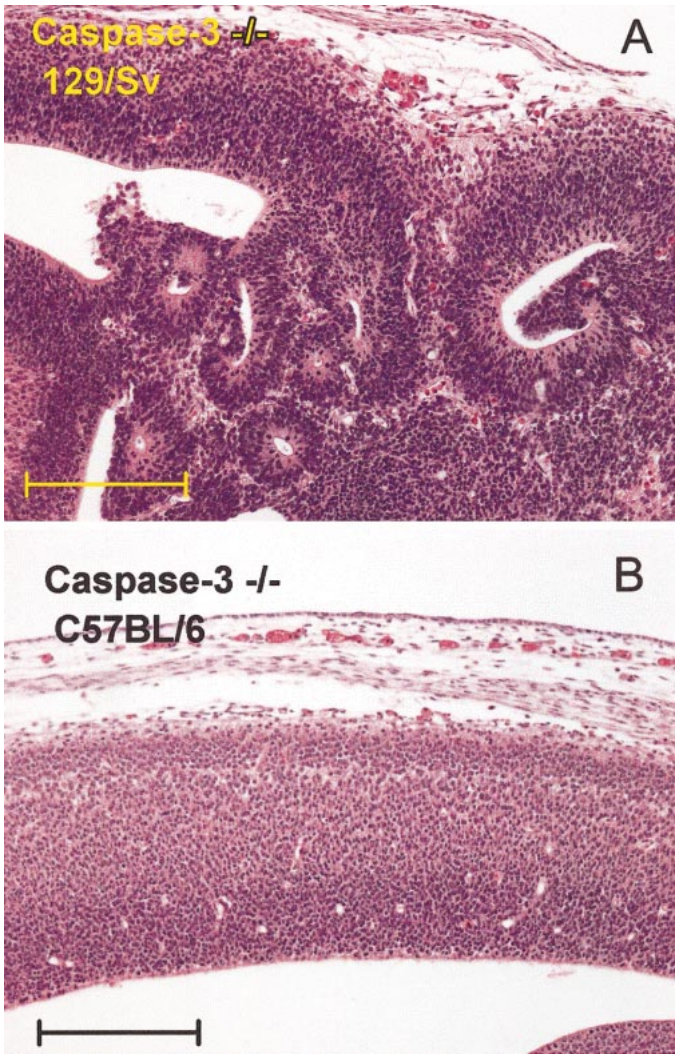

Fig. 1. Strain-specific brain pathology in caspase-3-deficient embryos. Hematoxylin and eosin-stained sagittal sections of caspase-3-deficient 129X1/SvJ (A) and C57BL/6J (B) embryos (E15) shows marked neural precursor cell hyperplasia and structural abnormalities in the 129X1/SvJ telencephalon but normal brain development in the C57BL/6J embryo. Scale bars = 100 μm.

dominant gene. An identical conclusion was obtained when survival of caspase-3-deficient F1 mice was assessed. We crossed *caspase-3*$^{-/-}$ C57BL/6J and *caspase-3*$^{+/-}$ 129X1/SvJ mice and obtained 18 mice that survived until weaning. Thirteen of these F1 mice were *caspase-3*$^{+/-}$ and only 5 were *caspase-3*$^{-/-}$, indicating that the frequency of F1 perinatal lethality is also intermediate between the 2 strains. Of the 5 *caspase-3*$^{-/-}$ F1 mice reaching adulthood, 4 were female and 1 male. Four of the 5 had some degree of gross abnormality (1 runt, 1 with marked hydrocephalus, and 2 with minor craniofacial abnormalities). Surviving male and female *caspase-3*$^{-/-}$ F1s were fertile. We crossed *caspase-3*$^{-/-}$ F1 mice with *caspase-3*$^{+/-}$ 129X1/SvJ animals and examined the

resultant *caspase-3*$^{-/-}$ E15–E18 mice. Of the 21 caspase-3-deficient mice analyzed, 12 were grossly abnormal and 18 had marked histopathological alterations similar to those observed in pure 129X1/SvJ *caspase-3*$^{-/-}$ mice (data not shown).

Genotoxic-Responsiveness Fails to Distinguish C57BL/6J and 129X1/SvJ *Caspase-3*$^{-/-}$ Neural Precursor Cells

129X1/SvJ *caspase-3*$^{-/-}$ embryonic mice display extensive telencephalic neural precursor cell expansion; in contrast, the *caspase-3*$^{-/-}$ C57BL/6J telencephalic neural precursor cell population appears normal. To determine if differential responsiveness of neural precursor cells to apoptotic death stimuli underlies the strain-specific effects of caspase-3 deficiency, we compared the death promoting effects of genotoxic injury (100 μM AraC and 10 Gy γ-IR) on freshly isolated E12.5 telencephalic neural precursor cells from control and *caspase-3*$^{-/-}$ embryos of both mouse strains. Similar to our previous study of genotoxic injury to caspase-3-deficient neural precursor cells derived from mice of a mixed genetic background (13), caspase-3-deficient cells from both pure genetic backgrounds showed only a slight and equivalent resistance to genotoxic-induced death (Fig. 2). This finding indicates that strain-specific neurodevelopmental abnormalities in caspase-3-deficient mice are not the result of differential neural precursor cell responsiveness to DNA damage.

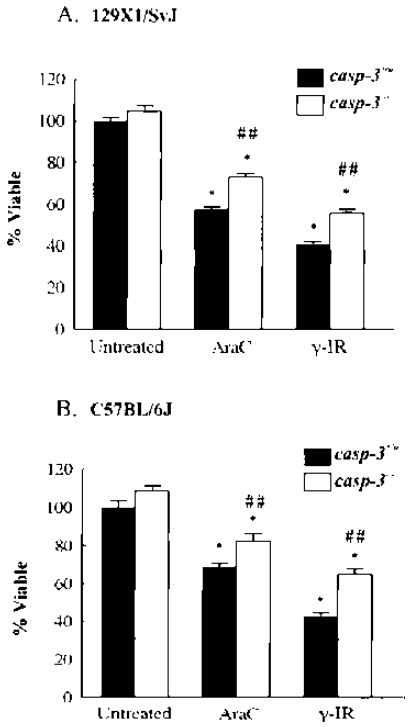

Fig. 2. DNA damage-induced neural precursor cell apoptosis does not require caspase-3 and is unaffected by genetic background. E12–E13 telencephalic cells were isolated from control (+/o includes +/+ and +/−) and *caspase-3* (−/−)129X1/SvJ (A) and C57BL/6J (B) embryos and treated with 100 μM AraC or 10 Gy γ-irradiation. Each data point represents mean ± SEM (n = 3–15 for the different groups). Data are presented as a percentage of untreated control cells. *$p < 0.001$ versus untreated of like genotype; ##$p < 0.001$ versus *caspase-3*$^{+/o}$ under similar treatment conditions (Tukey test).

DISCUSSION

Experiments over the last several years have demonstrated an important role for molecular regulators of programmed cell death in mammalian nervous system development (14). Targeted disruption of *caspase-3* has revealed that caspase-3 activation is critical for the generation of apoptotic cytological features and represents the commitment point to cell death in some neuronal apoptosis paradigms (15, 16). For example, caspase-3 deficiency protects Bcl-X_L-deficient neurons from premature death both in vivo and in vitro (17). Caspase-3-deficient mice were originally found to have a severe neurodevelopmental phenotype and we, and others, proposed that caspase-3 played a key role in neural precursor cell programmed cell death and brain morphogenesis (6, 17, 18). More recent studies and the findings reported here indicate an unexpected degree of complexity in the molecular pathways regulating mammalian nervous system development.

Caspase-3 deficiency on a pure C57BL/6J genetic background produced only minor neuropathological changes and caspase-3-deficient C57BL/6J mice survived into adulthood. In contrast, caspase-3-deficient 129X1/SvJ mice displayed exencephaly, marked neural precursor cell expansion, and died during the perinatal period. The majority of caspase-3-deficient F1 mice possessed a ''129X1/SvJ-like'' neuronal phenotype, indicating that the severity of the caspase-3-deficient phenotype is dependent upon one or more incompletely penetrant strain-specific genes. Strain-specific apoptotic responsiveness has been reported in a variety of neuronal death paradigms, although the genes controlling such differential responsiveness are largely unknown (19, 20).

Several possible explanations exist for the strain-specific effects of caspase-3 deficiency. Compensatory activation of other caspase effectors (e.g. caspase-6) in the caspase-3-deficient C57BL/6J, but not 129X1/SvJ nervous system, could selectively obviate the need for caspase-3 activation. Compensatory activation of caspase-6, caspase-7, and caspase-9 has been observed in caspase-3-deficient hepatocytes (21), although strain-specific neuronal compensation has not yet been demonstrated. An intriguing possibility is that strain-specific expression of endogenous inhibitors of apoptosis proteins (IAPs) underlies the variable caspase-3-deficient phenotype. The IAP family includes XIAP, cIAP-1, cIAP-2, and neuronal apoptosis inhibitory proteins (NAIPs) (22). Interestingly, there are significant differences in the *naip* gene array

between C57BL/6J and 129X1/SvJ mouse strains and a modifier gene that can delay onset of symptoms in a transgenic mouse model of amyotrophic lateral sclerosis maps to the murine *naip* gene locus (23, 24). Thus, strain-dependent inhibition of caspase activity could influence the severity of neurodevelopmental pathology in caspase-3-deficient mice. Finally, the observation that both caspase-9- and Apaf-1-deficient mice exhibit a variably severe phenotype, which is also likely to be strain-dependent, suggests that caspase-independent death pathways may also influence nervous system development. Relatively little is known about the molecular mediators of caspase-independent programmed cell death; however, such pathways exist in the nervous system and may provide an alternative mechanism for regulating neuronal cell death (25, 26). Regardless of the genetic underpinnings for the strain-specific effects of caspase-3 deficiency, our observations indicate caution in interpreting data generated from analyses of neuronal apoptotic death pathways in genetically mixed mice.

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