p53 deficiency fails to prevent increased programmed cell death in the Bcl-X_L-deficient nervous system

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Abstract

Bcl-X_L mice display a similar neurodevelopmental phenotype as *rb*, DNA ligase IV, and XRCC4 mutant embryos, suggesting that endogenous Bcl-X_L expression may protect immature neurons from death caused by DNA damage and/or cell cycle dysregulation. To test this hypothesis, we generated *bcl-x/p53* double mutants and examined neuronal cell death *in vivo* and *in vitro*. Bcl-X_L-deficient primary telencephalic neuron cultures were highly susceptible to the apoptotic effects of cytosine arabinoside (AraC), a known genotoxic agent. In contrast, neurons lacking p53, or both Bcl-X_L and p53, were markedly, and equivalently, resistant to AraC-induced caspase-3 activation and death *in vitro* indicating that Bcl-X_L lies downstream of p53 in DNA damage-induced neuronal death. Despite the ability of p53 deficiency to protect Bcl-X_L-deficient neurons from DNA damage-induced apoptosis *in vitro*, p53 deficiency had no effect on the increased caspase-3 activation and neuronal cell death observed in the developing Bcl-X_L-deficient nervous system. These findings suggest that Bcl-X_L expression in the developing nervous system critically regulates neuronal responsiveness to an apoptotic stimulus other than inadequate DNA repair or cell cycle abnormalities. *Cell Death and Differentiation* (2002) 9, 1063–1068. doi:10.1038/sj.cdd.4401067

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Abbreviations: AraC, cytosine arabinoside; DRG, dorsal root ganglion; H&E, hematoxylin and eosin; PBS-BB, phosphate buffered saline-blocking buffer; FCS, fetal calf serum; TSA, tyramide signal amplification

Introduction

Normal nervous system development requires the coordinated action of apoptotic stimuli with intracellular molecular

regulators of neuronal death.¹ The important role of neurotrophic factors in matching the size of developing neuronal populations with the extent of their post-synaptic targets has been well described.² However, only recently have other apoptotic stimuli been recognized as affecting mammalian nervous system development.^{3,4} The protein product of the tumor suppressor gene retinoblastoma (*rb*) is an important regulator of the cell cycle.^{5,6} The Rb-deficient nervous system displays numerous ectopic mitotic figures and many neurons undergoing apoptotic cell death. These findings suggest that failure of immature neurons to appropriately withdraw from the cell cycle could be an important stimulus for naturally-occurring neuronal cell death.⁷ Similarly, studies of mice with targeted gene-disruptions in *XRCC4* and *DNA ligase IV* have demonstrated that inadequate repair of DNA double strand breaks produces extensive death of neurons in the developing nervous system.^{8,9} The intracellular apoptotic effector mechanism for both cell cycle dysregulation and DNA damage appears to involve p53. p53 deficiency completely prevented the increased neuronal cell death observed in *XRCC4*-deficient and *DNA ligase IV*-deficient mice and it rescued Rb-deficient neurons in the central, but not peripheral, nervous system from premature death.^{10–13} Studies of 'double-knockout' mice have also shown involvement of ATM in the apoptosis of *DNA ligase IV*-deficient neurons and of caspase-3 in the death of Rb-deficient peripheral neurons.^{14,15} In total, these studies have provided insights into the potential stimuli and molecular pathways regulating naturally-occurring neuronal cell death.

Targeted gene disruption of the anti-apoptotic Bcl-2 family member, Bcl-X_L, resulted in extensive apoptotic death of immature neurons throughout the developing central and peripheral nervous systems in a pattern similar to that observed in *XRCC4*-, *DNA ligase IV*-, and Rb-deficient mice.¹⁶ Like Rb-deficient peripheral neurons, Bcl-X_L-deficient neurons were rescued from increased apoptosis by caspase-3 deficiency suggesting a possible link between cell cycle dysregulation, Bcl-X_L, and caspase-3-dependent death pathways.¹⁷ In a previous study of primary telencephalic neuronal cultures, we found increased caspase-3 activation and cell death in cytosine arabinoside (AraC)-exposed Bcl-X_L-deficient cells; in contrast, p53-deficient neurons were remarkably resistant to the effects of AraC.¹⁸ In an attempt to identify the apoptotic signals regulating naturally-occurring neuronal cell death and to define the molecular pathway of AraC-induced telencephalic neuron death, we generated *bcl-x^{-/-}/p53^{-/-}* mice and examined the effect of p53 deficiency on Bcl-X_L-deficient neurons *in vivo* and *in vitro*. Our data suggest that under normal developmental conditions, cell cycle dysregulation and inadequate DNA repair are relatively minor apoptotic stimuli in the central nervous system and that Bcl-

Figure 1 Quantification of caspase-3 activation and cell viability in AraC exposed control, p53-deficient, Bcl-X_L-deficient, and double-deficient telencephalic cells. Primary telencephalic neuron cultures were prepared from E12.5 control and homozygous mutant mice. After 48 h in a chemically-defined enriched media that promotes neuronal differentiation, cells were exposed to AraC (25 μ M) for an additional 48 h and the frequency of caspase-3 immunoreactive cells was assessed by CM1 immunostaining. Viability was determined by quantification of total nuclei with Hoechst 33,258 and non-viable nuclei by SYTOX Green labeling. Untreated cultures contained >93% viable cells and <3% caspase-3 immunoreactive cells and there was no difference in these measurements between genotypes (data not shown). AraC exposed control cells showed a significant increase in the number of caspase-3 immunoreactive cells (A) and a decrease in the percentage of viable cells (B). These effects were potentiated in *bcl-x*^{-/-}/*p53*^{+/o} cultures and attenuated in both *bcl-x*^{+/o}/*p53*^{-/-} and *bcl-x*^{-/-}/*p53*^{-/-} cultures. Similar results were obtained with 100 μ M AraC (data not shown). The percentage of viable cells in AraC exposed cultures was calculated for each genotype relative to untreated cells of the identical genotype. Heterozygous *bcl-x* and

X_L expression is critical for preventing immature neuron apoptosis caused by an undefined developmental signal(s) through a p53-independent pathway.

Results

p53 deficiency fails to rescue Bcl-X_L-deficient mice from embryonic lethality

To determine whether p53 deficiency is capable of preventing embryonic lethality in *bcl-x*^{-/-} mice, *bcl-x*^{+/o}/*p53*^{+/o} mice were interbred and surviving mice were genotyped at weaning. Genotypic analysis of 86 offspring revealed no *bcl-x*^{-/-} mice (expected Mendelian frequency is 1 in 16 assuming no embryonic lethality of either gene disruption or 1 in 13 assuming embryonic lethality of *bcl-x*^{-/-}/*p53*^{+/o} and *bcl-x*^{-/-}/*p53*^{+/o} mice; $P \leq 0.05$). Identical results were obtained when *bcl-x*^{+/o}/*p53*^{-/-} mice were interbred (i.e. zero *bcl-x*^{-/-} mice were observed in 24 genotyped offspring; expected frequency is one in four; $P \leq 0.05$). These findings suggest that p53 deficiency is incapable of preventing the hematopoietic and/or hepatic abnormalities that cause the embryonic lethality of Bcl-X_L-deficient mice. Bcl-X_L-deficient mice die around E13.5 and since p53 deficiency did not rescue the embryonic lethality of Bcl-X_L deficiency, we performed *in vitro* and *in vivo* studies of *bcl-x*^{-/-}/*p53*^{-/-} mice at E12.5.

p53 deficiency protects wild-type and Bcl-X_L-deficient telencephalic neurons from AraC-induced death *in vitro*

The E12.5 telencephalon consists predominantly of neural precursor cells which are unaffected by Bcl-X_L deficiency.^{16,17,19} We have previously shown that wild-type and Bcl-X_L-deficient telencephalic cell cultures grown in a chemically defined enriched medium for 48 h consist almost exclusively of post-mitotic neurons and show comparable, low levels of caspase-3 activation and apoptosis at baseline.^{19–21} We have also shown that addition of the nucleoside analog, AraC to telencephalic neurons *in vitro* results in extensive caspase-3 activation and cell death over the subsequent 24–48 h period.^{18,21} This effect is exacerbated in Bcl-X_L-deficient and attenuated in p53-deficient telencephalic cultures.^{18,21} p53 has both Bcl-2 family dependent and independent effects;^{22–24} we therefore examined the relationship between p53 and Bcl-X_L in untreated and AraC exposed (25 μ M) telencephalic neuron cultures prepared from control, *p53*^{-/-}, *bcl-x*^{-/-}, and *bcl-x*^{-/-}/*p53*^{-/-} E12.5 mice. In untreated cells, there was no significant difference in baseline viability (93–96%) or CM1 immunoreactivity (1.5–2.7%) between groups (data not shown). Forty-eight hours after exposure of wild-type telencephalic neurons to AraC, the percentage of

p53 telencephalic neurons responded similarly to wild-type neurons and therefore, data from wild-type and +/- cultures were pooled (+/o denotes +/- and +/- mice). Each data point represents mean $\pm$ S.E.M. ($n=31, 22, 5$, and 4 for *bcl-x*^{+/o}/*p53*^{+/o}, *bcl-x*^{+/o}/*p53*^{-/-}, *bcl-x*^{-/-}/*p53*^{+/o}, and *bcl-x*^{-/-}/*p53*^{-/-} groups, respectively). * $P \leq 0.05$ versus AraC treated *bcl-x*^{+/o}/*p53*^{+/o} cultures

cells with immunoreactive caspase-3 was dramatically increased and cell viability was markedly decreased (Figure 1A,B). Consistent with our previous studies, *p53*^{-/-} neurons showed a marked attenuation and *bcl-x*^{-/-} neurons a significant exacerbation of the effects of AraC on both caspase-3 activation and cell death (Figure 1A,B).^{18,21} Telencephalic neurons derived from *bcl-x*^{-/-}/*p53*^{-/-} embryos exhibited dramatic resistance to AraC-induced caspase-3 activation and death, equivalent to that observed in *bcl-x*^{+/-}/*p53*^{-/-} telencephalic cultures (Figure 1A,B). These findings suggest that AraC triggers a p53-dependent death pathway in telencephalic neurons that engages downstream Bcl-2 family mediated events including caspase-3 activation.

Increased apoptosis in the Bcl-X_L-deficient embryonic nervous system is unaffected by p53 deficiency

p53 deficiency rescues DNA Ligase IV- and XRCC4-deficient central and peripheral neurons from increased apoptosis during development and it selectively decreases neuronal apoptosis in the Rb-deficient central, but not peripheral, nervous system. To determine if p53 deficiency could similarly protect Bcl-X_L-deficient neurons from premature death *in vivo*, we examined control, *bcl-x*^{-/-}, *p53*^{-/-}, and *bcl-x*^{-/-}/*p53*^{-/-} mice on E12.5. H and E stained sections of E12.5 control mice showed occasional condensed, pyknotic, and/or fragmented nuclei indicative of naturally-occurring neuronal cell death in

the brainstem and spinal cord (Figure 2A). Apoptotic nuclear features were more common in Dorsal Root Ganglion (DRG) neurons and quantitation of such nuclei revealed approximately six apoptotic nuclei per 60 × microscopic field (Table 1). Similarly, immunodetection of activated caspase-3-like immunoreactivity in control embryos showed scattered positive cells in the developing central nervous system (Figure 3A), and approximately seven immunoreactive cells per 60 × microscopic field in the DRG (Table 1). p53 deficiency by itself had no effect on the frequency of activated caspase-3 immunoreactive cells or apoptotic nuclei in the E12.5 nervous system (Figures 2B, 3B and Table 1). In contrast, a dramatic

Table 1 Apoptosis in E12.5 dorsal root ganglia (cells/60 × microscopic field)

Genotype	Caspase-3 immunoreactive	Apoptotic nuclei
<i>bcl-x</i> ^{+/-} / <i>p53</i> ^{+/-}	7 ± 1	6 ± 2
<i>bcl-x</i> ^{+/-} / <i>p53</i> ^{-/-}	8 ± 1	5 ± 1
<i>bcl-x</i> ^{-/-} / <i>p53</i> ^{+/-}	48 ± 3*	32 ± 7*
<i>bcl-x</i> ^{-/-} / <i>p53</i> ^{-/-}	56 ± 5*	35 ± 6*

Bouin's fixed sagittal sections from paraffin-embedded E12.5 *bcl-x*^{+/-}/*p53*^{+/-}, *bcl-x*^{+/-}/*p53*^{-/-}, *bcl-x*^{-/-}/*p53*^{+/-}, and *bcl-x*^{-/-}/*p53*^{-/-} mice were H&E stained for quantification of apoptotic nuclei or immunostained with CM1 antiserum for detection of activated caspase-3-like reactivity. The mean number of positive cells and S.E.M. was determined for each group (*n*=5 per group). A gene dosage effect was not detected for either *bcl-x* or *p53* and therefore, data from +/+ and +/- embryos were pooled (+/- denotes +/- and +/- mice). *P* ≤ 0.05 versus *bcl-x*^{+/-}/*p53*^{+/-}.

Figure 2 p53 deficiency fails to affect programmed cell death in the wild-type or Bcl-X_L-deficient nervous system. H&E stained sections from the E12.5 spinal cord show only rare apoptotic nuclei in wild-type (A) and p53-deficient (B) mice (examples are indicated by arrows). In contrast, numerous apoptotic nuclei and phagocytic cells are seen in the Bcl-X_L-deficient (C) and Bcl-X_L-p53-double deficient (D) spinal cord. Scale bars, 20 microns

Figure 3 Activated caspase-3-like immunoreactivity in the nervous system is unaffected by p53 deficiency. Dual staining for caspase-3-like immunoreactivity (red) and nuclei (blue; bisbenzamide) in sections from the E12.5 brainstem show rare immunoreactive cells in wild-type (A) and p53-deficient (B) mice (examples indicated by arrows). A dramatic increase in caspase-3 immunoreactive cells is seen in the *bcl-x*^{-/-} brainstem (C) and there is no diminution of labeling in *bcl-x*^{-/-}/*p53*^{-/-} embryos (D). Scale bars, 50 microns

increase in caspase-3 activation and apoptotic death was observed in both the central and peripheral nervous systems of Bcl-X_L-deficient embryos (Figures 2C, 3C and Table 1). If the increased neuronal cell death observed in Bcl-X_L-deficient mice was the result of an apoptotic stimulus that engaged a p53-dependent death pathway, we would expect p53 deficiency to alleviate the effects of the *bcl-x* targeted gene disruption, as it does for *DNA ligase IV*, *XRCC4*, and, at least in the central nervous system, *rb* gene disruptions. We found that *bcl-x*^{-/-}/*p53*^{-/-} mice exhibited a neurodevelopmental phenotype identical to that of *bcl-x*^{-/-}/*p53*^{+/+} mice; i.e. they showed a marked increase in the number of activated caspase-3-immunoreactive and apoptotic neurons in both the central and peripheral nervous systems (Figures 2D, 3D and Table 1). In total, the inability of p53 deficiency to affect caspase-3 activation and neuronal apoptosis in either *bcl-x*^{+/+} or *bcl-x*^{-/-} embryos suggests that naturally-occurring neuronal cell death, at least at E12.5, is not critically regulated by a p53-dependent death stimulus nor is the increased neuronal apoptosis observed in the Bcl-X_L-deficient central nervous system due to inadequate repair of DNA strand breaks or cell cycle dysregulation since the death promoting effects of these stimuli are prevented by p53 deficiency.

Discussion

The interaction between p53 and the Bcl-2 family is complex, often exhibiting stimulus, cell type, and maturation-specific

effects.^{18,23–25} p53 and Bcl-2 family members may have entirely dependent or independent effects on cell death; thus, we examined the ability of p53 deficiency to rescue Bcl-X_L-deficient neurons from increased apoptotic death in an *in vitro* model of DNA damage-induced death and in the developing nervous system *in vivo*. Consistent with our previous *in vitro* studies of DNA damage-induced neuronal death, Bcl-X_L-deficient neurons showed increased, and p53-deficient neurons decreased, susceptibility to the death promoting effects of AraC, a known genotoxic agent.^{18,21} p53 deficiency completely blocked the increased apoptotic susceptibility of Bcl-X_L-deficient neurons to AraC and there was no difference in either caspase-3 activation or extent of death between *bcl-x*^{+/+}/*p53*^{-/-} and *bcl-x*^{-/-}/*p53*^{-/-} neurons. These findings suggest that Bcl-X_L lies downstream of p53 in the apoptotic pathway of DNA damage-induced death of telencephalic neurons. In combination with previous studies of DNA ligase IV- and XRCC4-deficient mice, the data are also consistent with the hypothesis that endogenous Bcl-X_L expression promotes immature neuron survival by allowing adequate time for DNA double strand break repair enzymes to function. To directly test this hypothesis, we generated *bcl-x*^{-/-}/*p53*^{-/-} mice and analyzed caspase-3 activation and neuronal cell death *in vivo*.

Bcl-X_L deficiency is lethal around gestational day 13.5 and *bcl-x*^{-/-} embryos exhibit extensive cell death in the developing hematopoietic and nervous systems. Genotyping of over 100 viable offspring from interbreeding *bcl-x*^{+/-}/*p53*^{-/-}

p53^{+/-} and/or *bcl-x*^{+/-}/*p53*^{+/-} mice failed to produce a single double-deficient mouse indicating that p53 deficiency does not rescue Bcl-X_L-deficient mice from embryonic lethality. Targeted disruptions of any of a variety of pro-apoptotic molecules, *bax*, *apaf-1*, *caspase-9*, and *caspase-3*, can rescue Bcl-X_L-deficient neurons from excess cell death; however, none of these gene disruptions rescue *bcl-x*^{+/-} embryos from premature lethality.^{17,18,20,26} Since the embryonic lethality of Bcl-X_L-deficient mice is thought secondary to increased hematopoietic and/or hepatocellular death, not neuronal death, we examined the central and peripheral nervous systems of control, *bcl-x*^{+/-}, *p53*^{+/-} and *bcl-x*^{+/-}/*p53*^{+/-} mice at gestational day 12.5.

Since Bcl-X_L, DNA ligase IV- and XRCC4-deficient embryos all show increased death of immature neurons,^{8,9} and p53 deficiency abolishes this effect in DNA ligase IV- and XRCC4-deficient mice,^{10,11} the ability of p53 deficiency to decrease immature neuron death in *bcl-x*^{+/-} embryos would support a role for Bcl-X_L in modulating neuronal responsiveness to naturally-occurring DNA damage and double strand break repair. In contrast, an inability of p53 deficiency to affect immature neuron death in *bcl-x*^{+/-} embryos would suggest that a p53-independent developmental stimulus and death pathway is more critically dependent on endogenous Bcl-X_L expression. Bcl-X_L-deficient E12.5 mice displayed markedly increased caspase-3 activation and apoptosis in immature neurons throughout the central and peripheral nervous systems while *bcl-x*^{+/-}/*p53*^{+/-} embryos appeared similar to wild-type littermates. Examination of *bcl-x*^{+/-}/*p53*^{+/-} E12.5 mice showed a neurodevelopmental phenotype identical with that observed in *bcl-x*^{+/-}/*p53*^{+/-} embryos. Double-deficient embryos exhibited extensive caspase-3 activation and neuronal cell death in both the central and peripheral nervous systems, equivalent to that observed in *bcl-x*^{+/-} embryos. These data indicate that excess death of Bcl-X_L-deficient neurons *in vivo* is not dependent on p53 expression nor is it likely to result from inadequate repair of DNA double-strand breaks.

In addition to DNA damage, cell cycle dysregulation is a possible apoptotic stimulus for developing neurons. Like *bcl-x*, *DNA ligase IV*, and *XRCC4* gene disruptions, *rb* gene disruption causes increased death of immature neurons in the developing central and peripheral nervous systems.^{5,6} pRb is a key regulator of the cell cycle and Rb-deficient neurons exhibit ectopic mitoses, impaired differentiation, and apoptotic death. Interestingly, the effects of Rb deficiency on central and peripheral neurons appear mediated by distinct apoptotic pathways. p53 deficiency rescues central nervous system Rb-deficient neuronal death, but not peripheral nervous system death, while caspase-3 deficiency rescues peripheral nervous system death but not central nervous system death in Rb-deficient mice.^{13,15} The inability of p53 deficiency to rescue either central or peripheral nervous system death in *bcl-x*^{+/-} embryos suggests that cell cycle dysregulation in the central nervous system is not a major apoptotic stimulus from which Bcl-X_L provides protection. In contrast, the ability of caspase-3 deficiency, but not p53 deficiency, to rescue both *bcl-x*^{+/-} and *rb*^{+/-} peripheral neurons from

apoptotic death suggests, but does not prove, that cell cycle dysregulation in the peripheral nervous system activates a p53-independent, Bcl-X_L- and caspase-3-dependent death pathway.

Neurotrophic factors and their receptors play a clear role in regulating survival of synapse-bearing neurons. However unlike the effect of Bcl-X_L deficiency, targeted disruptions of neurotrophic factor-related genes have produced relatively limited effects on immature neurons in the developing nervous system.^{27,28} It remains possible that other trophic molecules, such as FGF-2 or IGF-I, are limiting in the developing brain and that endogenous Bcl-X_L expression serves an anti-apoptotic function during the maturation of neural precursor cells into synapse-bearing neurons. Alternatively, still to be defined pro- and anti-apoptotic signals may be present in the nervous system that critically regulate expression of Bcl-X_L and/or its pro-apoptotic partner, Bax in immature neurons. Identification of such signals will be important for understanding mammalian nervous system development.

Materials and Methods

Generation of *bcl-x/p53* mutant mice

The generation and characterization of *bcl-x*^{+/-} mice has been described previously.¹⁶ *p53*^{+/-} mice were purchased from Taconic (Germantown, NY, USA). To produce mice deficient in both Bcl-X_L and p53, double heterozygote (*bcl-x*^{+/-}/*p53*^{+/-}) mice were interbred. Alternatively, *bcl-x*^{+/-}/*p53*^{+/-} mice were mated with either *bcl-x*^{+/-}/*p53*^{+/-} or *bcl-x*^{+/-}/*p53*^{+/-} mice. Endogenous and disrupted genes were detected by PCR analysis of tail DNA extracts as previously described.^{19,29} To determine whether the frequency of *bcl-x*^{+/-}/*p53*^{+/-} mice followed predicted Mendelian inheritance, χ^2 analysis of contingency tables was used.

Histology and immunohistochemistry

Pregnant mice were anesthetized with methoxyflurane and killed on gestational day 12.5 by cervical dislocation. Embryos (E12.5) were removed and tail and limb samples were taken for DNA extractions and PCR analyses. Whole embryos or embryos without their forebrains, which were used for telencephalic cell cultures, were placed in Bouin's fixative overnight at 4°C followed by three washes in 70% ethanol. Tissue was dehydrated, paraffin-embedded, and 4 μ m thick sagittal sections were cut. Sections were deparaffinized and hematoxylin and eosin (H&E)-stained as described previously.²⁰

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-3,³⁰ 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, USA), diluted 1:1000 in PBS-BB, for 1 h at room temperature. Immunostaining was detected using a tyramide signal amplification system (Perkin-Elmer Life Science Products, Boston, MA, USA). Tissue was counterstained with bisbenzimidazole (0.2 μ g/ml; Hoechst 33,258; Sigma, St. Louis, MO, USA) and examined with a Zeiss-Axioskop microscope equipped with

epifluorescence. The number of dorsal root ganglion cells with apoptotic nuclear features and CM1 immunoreactivity was assessed in multiple 60× magnification microscopic fields per mouse by an observer blinded to the embryo's genotype. Statistical significance was established using one way analysis of variance and the Tukey test.

Primary telencephalic cultures

E12.5 telencephalic cells were dissociated as previously described and plated in a chemically defined serum-free medium containing insulin, transferrin, selenium, progesterone, putrescine, glucose and glutamine¹⁹ followed by incubation at 37°C in humidified 5% CO₂/95% air atmosphere for 48 h. Cells were then placed in fresh medium containing 2% FCS with or without AraC (Sigma) for an additional 48 h. SYTOX green (500 nM; SG; Molecular Probes) was added to label dead cells. Cultures were then fixed with 4% paraformaldehyde (pH=7.4) for 20 min at 4°C. Activated caspase-3-like immunoreactivity was detected with CM1 antiserum and tyramide signal amplification techniques (TSA; Perkin-Elmer Life Science Products, Inc.) as previously described.³⁰ Cells were counterstained with bisbenzimidazole (Sigma) and visualized on a Zeiss-Axiovert microscope equipped with epifluorescence. Telencephalic cells from each embryo were plated in duplicate wells for each condition and the number of nuclei, CM1 and SG positive cells was counted at 40× magnification from multiple fields in each well. Approximately 150 to 200 nuclei were counted per well and significance was established using the Tukey test of multiple pairwise comparisons.

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