Bcl-x<sub>L</sub> is an Antiapoptotic Regulator for Postnatal CNS Neurons

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Bcl-x<sub>L</sub> is a death-inhibiting member of the Bcl-2/Ced9 family of proteins which either promote or inhibit apoptosis. Gene targeting has revealed that Bcl-x<sub>L</sub> is required for neuronal survival during brain development; however, Bcl-x<sub>L</sub> knock-out mice do not survive past embryonic day 13.5, precluding an analysis of Bcl-x<sub>L</sub> function at later stages of development. Bcl-x<sub>L</sub> expression is maintained at a high level postnatally in the CNS, suggesting that it may also regulate neuron survival in the postnatal period. To explore functions of Bcl-x<sub>L</sub> related to neuron survival in postnatal life, we generated transgenic mice overexpressing human Bcl-x<sub>L</sub> under the control of a pan-neuronal promoter. A line that showed strong overexpression in brainstem and a line that showed overexpression in hippocampus and cortex were chosen for analysis. We asked whether overexpression of Bcl-x<sub>L</sub> influences neuronal survival in the postnatal period by studying two injury paradigms that result in massive neuronal apoptosis. In the standard neonatal facial axotomy paradigm, Bcl-x<sub>L</sub> overexpression had substantial effects, with survival of 65% of the motor neurons 7 d after axotomy, as opposed to only 15% in nontransgenic littermates. To investigate whether Bcl-x<sub>L</sub> regulates survival of CNS neurons in the forebrain, we used a hypoxia-ischemia paradigm in neonatal mice. We show here that hypoxia-ischemia leads to substantial apoptosis in the hippocampus and cortex of wild-type neonatal mice. Furthermore, we show that overexpression of Bcl-x<sub>L</sub> is neuroprotective in this paradigm. We conclude that levels of Bcl-x<sub>L</sub> in postnatal neurons may be a critical determinant of their susceptibility to apoptosis.

Key words: apoptosis; axotomy; hypoxia-ischemia; Bcl-x<sub>L</sub>; Ced 9; Bax

Apoptosis is the most common form of physiological cell death and plays an important role in animal development and homeostasis, controlling cell numbers in both vertebrate and invertebrate tissues (Oppenheim, 1991; Raff et al., 1993). Remarkably, apoptosis is also a common response to neuronal injury, particularly in the neonatal period in avians and mammals (for review, see Elliott and Snider, 1998). Recently, several families of molecules that regulate apoptosis in different settings have been identified (for review, see Hengartner and Horvitz, 1994b; White, 1996). The Ced-9/Bcl-2 family of apoptosis regulators is composed of a large number of intracellular proteins with opposing effects in regulating cell death. Some family members, including Bcl-2 (Bakshi et al., 1985; Hengartner and Horvitz, 1994a) and Bcl-x<sub>L</sub> (Boise et al., 1993), function to inhibit apoptosis, whereas other members such as Bax (Oltvai et al., 1993), Bcl-x<sub>S</sub> (Boise et al., 1993), Bad (Yang et al., 1995), and Bak (Chittenden et al., 1995; Farrow et al., 1995; Kiefer et al., 1995) function to promote apoptosis. It appears that homodimerization and heterodimerization between the various death-promoting and death-inhibiting family members regulates the activation of caspases, which execute the cell death program (Korsmeyer, 1995; Yang et al., 1995; Reed, 1997). Although most of the analysis of these molecules has been done in extraneural tissues, there is now compelling evidence that their actions are quite general and include profound influences on neurons. In vitro studies have demonstrated that Bcl-2 and Bcl-x<sub>L</sub> can prevent apoptotic neuronal death induced by growth factor deprivation in primary neuronal cultures (Garcia et al., 1992; Allsopp et al., 1993; Frankowski et al., 1995; Gonzalez-Garcia et al., 1995; Greenlund et al., 1995). In contrast, overexpression of Bax and Bax in sympathetic neurons deprived of NGF accelerates apoptosis (Farrow et al., 1995; Easton et al., 1997). Bcl-2, Bcl-x<sub>L</sub>, Bax, and other members of the Bcl-2 family are expressed temporally and spatially in the nervous system in patterns compatible with a potential role as regulators of neuronal death in vivo (Gonzalez-Garcia et al., 1994; Krajewski et al., 1994; Merry et al., 1994; Frankowski et al., 1995; Parsadanian et al., 1995). Indeed, Bcl-2 overexpression in transgenic animals protects axotomized neonatal motor neurons from death and certain populations of developing neurons from naturally occurring cell death (Dubois-Dauphin et al., 1994; Martinou et al., 1994; Farlie et al., 1995).

The physiological importance of these molecules for neuronal survival has been demonstrated recently by gene targeting. The phenotype of Bcl-x<sub>S</sub> null mutants is striking. Mice die at approximately embryonic day 13. Extensive apoptotic cell death is evident in postmitotic immature neurons of developing brain, spinal cord, and dorsal root ganglia (Motyama et al., 1995). The consequences of the Bcl-2 null mutation are less dramatic, but several populations of neurons in the PNS are partially depleted (Michaelidis et al., 1996). Finally, deletion of the Bax gene also has dramatic but opposite effects. In both sympathetic and motor neuron populations, cell numbers in newborn mice are increased,
is in contrast to Bcl-2, is that Bcl-xL expression is maintained at embryonic development, but functions of Bcl-xL in the postnatal neurons in vitro their targets by axotomy, respectively. Knock-out mice survive NGF deprivation and disconnection from their targets by axotomy, respectively.

Bcl-xL is the dominant inhibitor of neuronal apoptosis during the early postnatal period. Studies involving Bcl-xL knock-out mice demonstrate that Bcl-xL overexpression prevented apoptosis of cortical and hippocampal neurons in a hypoxia–ischemia paradigm in neonatal mice. Our results demonstrate that Bcl-xL is a powerful regulator of neuronal apoptosis in the CNS during the early postnatal period in vivo.

A striking feature of the antiapoptosis regulator Bcl-xL, which is in contrast to Bcl-2, is that Bcl-xL expression is maintained at high levels throughout the postnatal CNS (Gonzalez-Garcia et al., 1994; Merry et al., 1994; Gonzalez-Garcia et al., 1995; Parsadanian et al., 1995). This persistent expression parallels the time frame when neurons lose their survival dependence on neuronal growth factors. An attractive hypothesis is that relative levels of Bcl-xL compared with levels of the prodeath regulator Bax, are important in downregulating survival dependence on growth factors. A first step in addressing this hypothesis is to determine whether Bcl-xL can regulate neuronal apoptosis in the postnatal CNS. Studies involving Bcl-xL knock-out mice demonstrate that Bcl-xL is the dominant inhibitor of neuronal apoptosis during embryonic development, but functions of Bcl-xL in the postnatal period cannot be determined in these mice because of early lethality. Currently, there are no data addressing whether Bcl-xL can regulate neuronal apoptosis in the postnatal brain.

To address functions of Bcl-xL in vivo, we generated transgenic mice overexpressing human Bcl-xL in neurons. We found that increased expression of Bcl-xL prevented apoptosis in the standard neonatal facial motor neuron axotomy model. We also found that Bcl-xL overexpression prevented apoptosis of cortical and hippocampal neurons in a hypoxia–ischemia paradigm in neonatal mice. Our results demonstrate that Bcl-xL is a powerful regulator of neuronal apoptosis in the CNS during the early postnatal period in vivo.

**Figure 1.** Generation and characterization of Bcl-xL transgenic mice. A, Schematic presentation of the Bcl-xL transgene construct: RI, EcoRI; N, NotI; S, SacI; Sa, SalI; X, XhoI; Xb, XbaI. B, Southern blot analysis of the different transgenic lines. The 6 kb fragment corresponds to the endogenous Bcl-xL gene. The 0.8 kb fragment corresponds to Bcl-xL transgene. Lines 7193, 7199, and 7194 had the highest copy numbers and were used for further analysis.

**MATERIALS AND METHODS**

*Generation of transgenic mice.* In the first step of making our transgene construct, an 850 bp XhoI–SacI fragment, containing a portion of the SV40 small T antigen with an intron and polyadenylation signal, was cloned into Bluescript SK plasmid (Stratagene, La Jolla, CA), containing an 0.8 kb EcoRI fragment of the Bcl-xL cDNA (Boise et al., 1993). This resulted in a plasmid termed pBclxL-pA1. In the second step, the plasmid p253Not (a gift from Dr. F. Miller, Montreal Neurological Institute), containing a 1.1 kb SalI–XhoI fragment of the Tal a-tubulin promoter, was digested with XhoI, blunt-ended by Klenow enzyme treatment, further digested with SalI, and cloned into plasmid pBclxL-pA1 digested with SalI and EcoRV. The resulting plasmid pTel-BclxL-pA6 was digested with NotI and SacI. The 2.75 kb transgene fragment was then eluted from an agarose gel and used for pronuclear injection (strain B6/CBA). Integration of the transgene into the mouse genome was determined by PCR and Southern blot analysis on genomic DNA isolated from mouse tails.

*Southern blot and PCR analysis.* Eight micrograms of genomic DNA extracted from mouse tail were digested with EcoRI, separated in 0.8% agarose gel, and transferred to a positively charged nylon membrane (Boehringer Mannheim, Indianapolis, IN) by standard capillary blotting. A 0.8 kb digoxigenin (DIG)-labeled PCR product, corresponding to Bcl-xL cDNA, was used as a probe for hybridization in DIG Easy Hyb solution (Boehringer Mannheim). The blots were hybridized at 42°C overnight and then washed for 1 hr in 2x SSC and 0.1% SDS at 65°C and 1 hr in 0.1× SSC and 0.1% SDS. For detection we used the DIG luminescent detection kit (Boehringer Mannheim) following the manufacturer’s instructions. The filters were exposed to Kodak (Rochester, NY) X-OMAT AR film. The films were scanned using a densitometer, and transgene copy number was determined by comparison of the signal intensities between the endogenous and transgene bands.

Integration of the transgene was analyzed routinely by PCR using the 5'-oligo (5'-CTGAATGACACCTAGAGCGTGG-3') and the 3'-oligo (5'-GAATGTTGAGGTCCAGCAGATGC-3'). PCR was performed in a 50 μl reaction, containing 1× PCR buffer, 1.5 mM MgCl2, a 150 μM concentration of each dNTP, a 0.3 μM concentration of each primer, and 1.25 U of Taq-polymerase (Life Technologies, Gaithersburg,
MD), in the following conditions: 94°C for 1 min, 64°C for 1 min, and 72°C for 1 min, for 35 cycles, followed by a 7 min extension at 72°C.

In situ hybridization. Transcription of the construct shown in Figure 1A produces an mRNA containing the coding regions of both human Bcl-xL and a portion of the SV40 small T antigen. Thus, for in situ hybridization, two different antisense riboprobes were used. The first one corresponds to the coding region of human Bcl-xL. This probe recognizes both endogenous (mouse) and transgenic (human) Bcl-xL mRNA. The second probe corresponds to the SV40 small T antigen and recognizes only transgenic Bcl-xL mRNA. F1 offspring from different transgenic lines were used for in situ hybridization. Mice were anesthetized with halothane and quickly decapitated, and the brains and spinal cords were frozen on dry ice. Cryostat sections (12–20 μm) were cut, thaw-mounted onto Super Frost Plus slides (Fisher Scientific, Houston, TX), and stored at 220°. On the day of hybridization, slides were thawed, and hybridization was performed as described previously (Wright et al., 1995).

Facial nerve axotomy and motor neuron analysis. F1 offspring from matings between Tal-Bcl-xL transgenic animals and CF1 wild-type animals were killed 1 week after the axotomy with an overdose of sodium pentobarbital and fixed by intracardiac perfusion with 4% paraformaldehyde. The brains were removed, embedded in paraffin, sectioned at 12 μm, and stained with cresyl violet. Six wild-type and six transgenic animals were studied. The facial nucleus was identified, and every fourth section was evaluated. To ensure the accuracy of cell counts in the facial nucleus, we compared the numbers of cells counted with the physical dissector with the counts obtained using standard profile methods. No significant difference in the number of neurons in the facial nucleus was found between profile counts of the nucleoli and the physical dissector method. Therefore, counts of nucleoli that came into focus through the plane of the section were used as the primary method of determining cell number. Student’s t test was used to compare the mean number of surviving motor neurons between wild-type control and Bcl-xL-overexpressing animals.

Immunohistochemistry, terminal deoxynucleotidyl transferase-mediated deoxy-UTP nick-end-labeling staining, and electron microscopy. Transgenic and control embryos were perfused in PBS followed by 3% paraformaldehyde with 15% picric acid in 0.1 M phosphate buffer, pH 7.4. The brains were immediately removed and cryoprotected in 30% sucrose/PBS. Tissue was then frozen in O.C.T. compound and sectioned coronally on a cryostat at 12 μm. After rehydration in PBS, sections were blocked for 30

Figure 2. The Bcl-xL transgene is expressed at high levels in the facial motor nucleus of line 7193. A, Section through the brainstem of a transgenic mouse hybridized with a probe for Bcl-xL. This probe detects endogenous and transgenic Bcl-xL mRNA, both of which are expressed throughout the brainstem and facial nucleus. B, Adjacent section from the same animal as in A, hybridized with the SV40 probe, which detects only the transgene-derived Bcl-xL mRNA. Note the high levels of expression of SV40 in motor neurons of the facial nucleus (arrows). C, Brainstem section of a wild-type littermate hybridized with the SV40 probe. Note that no signal was detected in the facial nucleus. Scale bar, 0.5 mm.
min in Superblock buffer (Pierce, Rockford, IL) with 1% porcine gelatin, 2% normal horse serum, and 0.3% Triton X-100. A rabbit polyclonal antibody to the receptor tyrosine kinase Ret (kindly provided by Qiao Yan, Amgen) was diluted 1:1500 in the blocking solution and incubated overnight at 4°C. The following day, sections were rinsed in PBS and incubated with biotinylated secondary antibody (Vector Laboratories, Burlingame, CA) diluted 1:150 in the blocking solution for 30 min at room temperature. Slides were incubated subsequently with HRP-labeled streptavidin (ABC kit, Vector). Primary antibodies were visualized with 3,3'-diaminobenzidine as the HRP substrate. Slides were then rinsed, dehydrated through graded alcohols, and coverslipped. Terminal deoxynucleotidyl transferase-mediated deoxy-UTP nick end labeling (TUNEL) was performed on P7 mouse brain tissue according to the manufacturer’s instructions (Oncor, Inc.). Electron microscopy on P7 mouse brain was performed as described previously (Golden et al., 1993).

RESULTS

Generation of transgenic mice

To overexpress human Bcl-x<sub>L</sub> in neurons we used the α-tubulin (Tα1) promoter, which has been well characterized previously using a lacZ reporter gene in transgenic mice. In most lines, this promoter drives lacZ at high levels in neurons during embryonic period. The pups were then placed in containers through which humidified 8% oxygen and balance nitrogen flowed for the next 1 hr. The contains were partially submerged in a 37°C water bath to maintain normothermia during this period. After retrieval from the hypoxic chamber, pups were returned to their dam. Mortality during surgery or the exposure to hypoxia was ~20%; however, mortality did not differ between the control and transgenic animals. Seven days after treatment, brains were processed, and tissue loss caused by hypoxic–ischemia was determined by calculating the amount of surviving tissue in the damaged versus the undamaged hemisphere in coronal sections exactly as described previously (Holtzman et al., 1996; Cheng et al., 1997).

Figure 3. The Bcl-x<sub>L</sub> transgene is expressed at high levels in the forebrain of line 7194. A. Section through the forebrain of a transgenic mouse hybridized with a probe for Bcl-x<sub>L</sub>. This probe detects both the endogenous and transgene-derived Bcl-x<sub>L</sub>. B. Adjacent section from the same brain as in A, hybridized with the SV40 probe, which detects only transgene-derived Bcl-x<sub>L</sub> mRNA. Note the high levels of transgene expression in the cerebral cortex (arrows) and thalamic nuclei. Also note the high expression in CA1, CA2 and CA4 regions of the hippocampus, and the minimal expression in the dentate and CA3 regions. Scale bar, 1 mm.
development and at lower, but readily detectable, levels postnatally (Gloster et al., 1994; Majdan et al., 1997). After injection of the transgene construct (Fig. 1A), 12 founder mice were obtained of 83 screened. Transgene copy number was determined by Southern blot analysis (Fig. 1B). Transgene copy number differed from line to line and varied from 2 to 60 copies.

**Analysis of Bcl-x<sub>L</sub> expression in different transgenic lines**

The expression pattern of the transgene was analyzed in F1 offspring of all transgenic lines using in situ hybridization. The cDNA sequences of human and mouse Bcl-x<sub>L</sub> are highly con-

**Figure 4.** Bcl-x<sub>L</sub> overexpression protects motor neurons from axotomy-induced cell death. A, Ret-labeled motor neurons in the facial nucleus of a nonaxotomized wild-type mouse. Note the labeling in the medial and lateral (circled) portion of the nucleus. Inset, High-magnification view of Ret labeling in wild-type neurons. B, Ret-labeled motor neurons in the facial nucleus of an axotomized wild-type mouse. Cells in the lateral portion of the nucleus have degenerated (circled), and Ret is no longer detectable except in the medial portion of the nucleus, which is not affected by this lesion paradigm. C, Ret-labeled motor neurons in the facial nucleus of an axotomized transgenic Bcl-x<sub>L</sub> mouse (line 7193). Many lateral motor neurons survive axotomy (circled), although they are reduced in size. Inset, High-magnification view of Ret labeling in the rescued lateral lateral motor neurons. (Compare the size of motor neurons in the inset in C with the inset in A.) D, Cresyl violet-stained motor neurons in the facial nucleus of an axotomized transgenic Bcl-x<sub>L</sub> mouse. E, High-magnification bright-field view of facial motor neurons 7 d after axotomy in transgenic line 7194. Arrows indicate rescued motor neurons. F, Dark-field view of the same section as in E, labeled with the SV40 probe to detect transgene-derived sequences. Note the high degree of correlation between the rescued motor neurons shown in E and the expression of transgene-derived Bcl-x<sub>L</sub> shown in F (arrows). Scale bars: A–D, 100 μm; E–F, insets, 25 μm.
served, and the Bcl-xL riboprobe that we used detected both endogenous and transgenic Bcl-xL mRNA. To distinguish between these, we designed a riboprobe corresponding to the SV40 small T antigen mRNA, which is included in the transcript derived from the transgene construct and therefore allowed us to detect specific expression of the transgene.

In situ hybridization results showed that most of the transgenic lines expressed Bcl-xL mRNA, but that expression patterns were highly variable. The SV40 small T antigen antisense probe allowed a definitive analysis of transgene expression, because no signal above background was detected by in situ hybridization when wild-type neuronal tissue was hybridized (Fig. 2C). Lines 7193, 7194, and 7199 were chosen for detailed analysis of Bcl-xL transgene expression. In line 7193, Bcl-xL was expressed at particularly high levels in cerebellum and in motor neurons of the facial nucleus at P7 (Fig. 2A,B). In line 7194, Bcl-xL was expressed in many neurons of the cerebral cortex, as well as in most neurons in thalamic nuclei at P9 (Fig. 3A,B). The transgene was also expressed at high levels in neurons in the CA1, CA2, and CA4 regions of the hippocampus, with less expression in CA3 and dentate gyrus (Fig. 3B). A minority of neurons in the facial motor nucleus also expressed the Bcl-xL transgene in this line.

Bcl-xL overexpression rescues facial motor neurons from axotomy-induced cell death

To address the question of whether Bcl-xL overexpression may influence motor neuron survival, we transplanted neonatal facial nerves, which causes massive death of facial motor neurons in wild-type animals. The right facial nerve was transected at P2 in F1 offspring from different Tα1-Bcl-xL transgenic lines and littermate controls. One week after axotomy, the animals were killed. It was obvious in initial experiments that many cells were present in the facial nucleus on the axotomized side in Bcl-xL transgenic mice. To verify that the cells in the axotomized facial motor nucleus were motor neurons, we stained sections with an antibody to the glial cell line-derived neurotrophic factor receptor Ret, which is expressed selectively by motor neurons in spinal cord and brainstem (Trupp et al., 1997). In nonaxotomized wild-type control animals, labeling with this antibody demarcates the facial motor pool clearly (Fig. 4A). In axotomized wild-type animals, only occasional cells were labeled in the lateral portion of the nucleus (Fig. 4B). It should be noted that although 15% of facial motor neurons remain when the entire nucleus is counted, virtually all surviving neurons are in the medial subnucleus, which projects axons to the auricular musculature and are unlesioned in this paradigm (Dubois-Dauphin et al., 1994). In striking contrast to controls, in the axotomized transgenic animals, cells throughout the facial nucleus were labeled (Fig. 4C), indicating that the surviving cells are in fact motor neurons and not interneurons or glia.

Counts of facial motor neurons in Nissl-stained serial sections 1 week after axotomy in line 7193, in which most facial motor neurons expressed the Bcl-xL transgene, demonstrated that an average of 65% of motor neurons survived 1 week after nerve lesion (Figs. 4C,D, 5). The surviving cells were reduced in size (Fig. 4, compare A,C, insets), indicating that motor neurons disconnected from target tissue by axotomy underwent considerable atrophy. Counts of the facial nucleus in unlesioned transgenic animals compared with unlesioned controls revealed no significant difference in the number of motor neurons (Fig. 5). This indicates that although injury-induced apoptosis is suppressed, naturally occurring cell death in the embryonic period was not prevented.

In lines 7194 and 7199, fewer facial motor neurons expressed transgenic human Bcl-xL, and fewer motor neurons survived axotomy (e.g., in 7199, ~33% of the motor neurons survived 1 week after the lesion; n = 3). The correlation between transgene expression and neurons that survive axotomy was studied in line 7194. For example, Figure 4, E and F, shows motor neuron survival (bright field) and transgene expression (dark field) of the same section in the lateral region of the facial nucleus 1 week after axotomy. As indicated by the arrows, each of the surviving facial motor neurons expresses the Bcl-xL transgene.

Neonatal hypoxic–ischemic insult results in apoptosis of forebrain neurons

As opposed to some hypoxia–ischemia paradigms in the adult CNS in which many cells may die via necrosis (Brown and Brierley, 1972), recent data suggest that after hypoxic–ischemic injury to the neonatal brain, many cells die via apoptosis (Ferrer et al., 1994; MacManus et al., 1994; Mehmet et al., 1994; Hill et al., 1995). To examine this issue further, we used a well characterized model of neonatal hypoxia–ischemia (modified Levine procedure), which results in unilateral hypoxic–ischemic brain injury (Rice et al., 1981; Johnston, 1983; Ferriero et al., 1995; Holtzman et al., 1996). We found that unilateral carotid ligation and exposure to 8% O2 for 2.5 hr in P7 rats or 1 hr in P7 mice result in significant damage to the hemisphere ipsilateral to carotid ligation and no damage to the contralateral hemisphere (Ferriero et al., 1995; Holtzman et al., 1996; Cheng et al., 1997). In P7 mice, analysis of the brain ipsilateral to carotid ligation revealed that in cortex, hippocampus, and striatum, some nuclei begin to label with the TUNEL method at 6 hr after the termination of hypoxic exposure (Fig. 6B). The number of TUNEL-positive nuclei increased after this time and reached a peak by 18 hr (Fig. 6C,D). Only occasional TUNEL-positive cells were seen contralateral to carotid ligation, but the number of these cells was similar to that seen in normal P7 mice (data not shown). That the TUNEL labeling was nuclear was demonstrated 12 hr after ischemia by staining sections with hematoxylin and eosin (Fig. 6E,F).
Whereas the TUNEL method is a sensitive indicator of apoptosis, it is not specific. We therefore performed electron microscopy. We found that there were occasional apoptotic cells in cortex and hippocampus 6 hr after hypoxic treatment ipsilateral to carotid ligation. By 12 hr, there were many cells in both hippocampus and cortex with condensed chromatin and cell shrinkage in every section of ischemic tissue examined (Fig. 7A). Although we cannot rule out that some necrosis occurs, our findings corroborate those of others and suggest that many cells are dying via apoptosis in this model.

**Bcl-xL overexpression protects neonatal mouse brain from hypoxic–ischemic insult**

We were interested in whether Bcl-xL overexpression could prevent neuronal cell death in this model that favors apoptosis in the CNS. Our in situ hybridization results showed that in line 7194, the Bcl-xL transgene is expressed at high levels during the first 2 weeks after birth in the cerebral cortex and hippocampus, regions that are damaged significantly in this hypoxia–ischemia paradigm. Based on these in situ hybridization results, we tested whether
overexpression of Bcl-xL would protect against neonatal hypoxic–ischemic brain injury.

P7 mice from transgenic line 7194 and wild-type littermates received unilateral (left) carotid artery ligation and were exposed to 8% oxygen for 1 hr. Brains were analyzed 1 week later for extent of tissue damage. We found that the amounts of tissue loss (ipsilateral to carotid ligation) in the striatum, cortex, and hippocampus of wild-type animals were 40.1, 30.8, and 52.3%, respectively. In transgenic animals from line 7194, the amounts of tissue loss in striatum, cortex, and hippocampus were 24.6, 11.9, and 31.9%, respectively. Thus, there was 61.5% less damage in the cortex, 39% less damage in the hippocampus, and 38.5% less damage in the striatum of animals overexpressing Bcl-xL (Fig. 8). All of these changes were statistically significant.

DISCUSSION

Bcl-xL prevents axotomy-induced apoptosis in the early postnatal period

Using the pan-neuronal promoter Tal α-tubulin, we overexpressed human Bcl-xL in neurons. As expected from previous studies, the pattern of transgene expression was highly variable among different lines (Gloster et al., 1994; Majdan et al., 1997). Overexpression of Bcl-xL protected facial motor neurons from axotomy-induced cell death in the neonatal period. The degree of protection varied between the different lines and was correlated with the percentage of facial motor neurons that expressed the transgene. Because all facial motor neurons express endogenous Bcl-xL in the postnatal period presumably to some degree, the absolute level of expression must be critical in regulating susceptibility to apoptosis.

It is interesting to compare our results with Bcl-xL overexpression with other studies that have modulated levels of expression of genes encoding Bcl-2 family members. Overexpression of Bcl-2 under the neuron-specific enolase (NSE) promoter produced results that were similar to those reported here (Dubois-Dauphin et al., 1994). In two of those lines, the majority of motor neurons were protected from axotomy-induced cell death. Thus, an interesting implication of our study is that from a pharmacological standpoint, Bcl-xL and Bcl-2 appear to be interchangeable in regulating motor neuron survival in vivo. In mice that lack the proapoptotic regulator Bax, profound saving of motor neurons was also observed after neonatal facial nerve axotomy (Deckwerth et al., 1996). This latter finding supports the idea that proapoptotic and antiapoptotic Bcl-2 family members interact to regulate susceptibility of postnatal motor neurons to apoptosis.

It is perhaps surprising that there was no reduction in the amount of naturally occurring cell death in the facial motor nucleus. Studies of Bcl-2 overexpression using the NSE promoter showed that lines were highly variable in their ability to prevent naturally occurring cell death related presumably to the developmental age of onset of transgenic Bcl-2 expression (Martinou et al., 1994). The known properties of the Tal α-tubulin promoter suggest that it should be active during early development (Gloster et al., 1994). However, because we were interested in postnatal responses, we did not characterize expression patterns of transgenic Bcl-xL during embryogenesis. Thus, we do not know whether the lack of increase in neuronal numbers is attributable to a relatively late onset of transgenic Bcl-xL expression or expression in the embryo that is below the level required to prevent naturally occurring cell death.

Bcl-xL reduces damage caused by neonatal hypoxia-ischemia

Endogenous Bcl-xL is widely expressed in the nervous system in both embryonic and postnatal life (Gonzalez-Garcia et al., 1994; Merry et al., 1994; Gonzalez-Garcia et al., 1995; Parsadanian et al., 1995). To determine whether regulation of apoptosis in vivo by Bcl-xL generalized to cells other than motor neurons, we sought a model of apoptosis of forebrain neurons. Hypoxic–ischemic injury in the adult brain generally leads to necrotic cell death within the “core” of infarcted tissue. However, previous studies have suggested that even severe hypoxic–ischemic brain injury in the...
neonatal period can lead to apoptosis. We have confirmed and extended these earlier studies (Ferrer et al., 1994; Mehmet et al., 1994; Hill et al., 1995). Carotid ligation in wild-type neonatal mice followed by exposure to hypoxia resulted in a marked amount of DNA damage in cortex, hippocampus, and striatum, as revealed by TUNEL staining. Because TUNEL may not be specific for apoptotic cell death, we performed ultrastructural analysis. Abundant nuclei with changes that resembled apoptosis were seen by EM at 6 and 12 hr after injury. Analogous results have been reported recently that show that injection of glutamate receptor agonists that trigger necrosis in mature rat brain leads to apoptosis in neonatal brain (Portera-Cailliau et al., 1997). Further evidence that this neonatal hypoxic–ischemic injury-induced death has a prominent apoptotic component is provided by the fact that it can be prevented almost completely by injections of BDNF into the lateral ventricle in neonatal rats (Cheng et al., 1997). Interestingly, BDNF is only marginally effective in reducing damage caused by ischemia later in CNS development in the same model (Cheng et al., 1997).

Overexpression of Bcl-xL had impressive survival-promoting effects in neonatal hypoxic–ischemia-induced brain injury. There was ~50% reduction in the volume of damage in the hippocampus, cortex, and striatum in mice that overexpress Bcl-xL. It is possible that the protection would have been even greater if Bcl-xL was expressed in all cells (e.g., glia and endothelial cells) and not limited to neurons. Thus, Bcl-xL appears to be effective in regulating death of cells contained completely within the CNS as well as those with peripheral projections (facial motor neurons). This generality of Bcl-xL action is interesting in light of recent demonstrations that PNS and CNS neurons exhibit differing patterns of survival dependence related to neurotrophins and neural activity (Meyer-Franke et al., 1995).

These observations may have useful clinical implications. This experimental paradigm is a rodent model of hypoxic brain injury, which occurs in the perinatal period in humans and leads to conditions such as cerebral palsy. Once such an insult has occurred, blood supply and oxygen levels usually can return to normal. Thus, if molecules such as Bcl-xL can protect cells against death caused by a “transient” insult, it may provide enough protection for long-term cellular survival and function once the brain environment returns to baseline. An exciting possibility is that factors that augment Bcl-xL actions may be useful as treatments after neonatal hypoxic–ischemic insults.

Potential interactions of Bcl-xL and Bax in regulating postnatal neuron survival

A striking feature of gene expression in Bcl-2 family members is that Bcl-2 is downregulated rapidly during development in all except a few neuronal populations. In contrast, Bcl-xL is either maintained or upregulated during development and into adulthood (Gonzalez-Garcia et al., 1994; Merry et al., 1994; Gonzalez-Garcia et al., 1995; Parsadanian et al., 1995). Similarly, although the precise developmental time frame is less clear, Bax is also expressed at appreciable levels by mature neurons (Krajewski et
The levels of Bcl-xL and Bax in the postnatal period are related to powerful stimuli of axonal injury and hypoxia–ischemia. After injury, susceptibility to apoptosis markedly in response to the powerful stimuli of axonal injury and hypoxia–ischemia. Cheng Y, Gidday JM, Yan Q, Shah AR, Holtzman DM (1997) Marked overexpression of Bax, rapidly leads to death of postnatal sympathetic ganglion neurons in vitro that are normally growth factor-independent (Eauston et al., 1997). Taken together, these results support the idea that the Bcl-xL/Bax ratio is critical in setting a threshold for neuronal apoptosis in the postnatal CNS.

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