

Dynamics of expression of apoptosis-regulatory proteins Bid, Bcl-2, Bcl-X, Bax and Bak during development of murine nervous system

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Received 15.11.00; revised 20.4.01; accepted 10.7.01

Edited by DR Green

Abstract

We have used immunohistochemistry and immunoblotting to examine the expression of Bid and four other Bcl-2 family proteins (Bcl-2, Bcl-X, Bax and Bak) in the developing and adult murine central nervous system (CNS). Bid protein is widespread in embryonic and postnatal brain, and its expression is maintained at a high level late into the adulthood. Bid is expressed both in the germ disc, early neural tube, proliferating stem cells of ventricular zones, and in postmitotic, differentiated neurons of the developing central and peripheral nervous system. As the differentiation proceeds, the neurons express higher levels of Bid than the stem cells of the paraventricular zone. Both in embryonic and postnatal life, Bid protein is present in the most vital regions of brain, such as the limbic system, basal ganglia, mesencephalic tectum, Purkinje cells in cerebellum, and the ventral columns of spinal cord. The p15 cleaved form of Bid was detectable in the brain specimens at fetal stages of development, consistent with caspase-mediated activation of this pro-apoptotic Bcl-2 family protein. Among the Bcl-2 family proteins only Bid and Bcl-X_L continue to be expressed at high levels in the adult brain.

Cell Death and Differentiation (2002) 9, 145–157. DOI: 10.1038/sj/cdd/4400934

Keywords: neuronal apoptosis; CNS; development; Bid; Bcl-2 family

Abbreviations: CNS, central nervous system; PNS, peripheral nervous system; DRG, dorsal root ganglia; E4.5–8/TS6–12, etc., staging of mouse development according to time after conception and Theiler Stages¹; ME12, mouse embryo at day 12.

Introduction

Programmed cell death has long been recognized as an important process in normal development of the vertebrate

nervous system, with as many as 50 to 85% of neurons dying in some regions.² Members of the Bcl-2 family play critical roles during the development of the nervous system as well as in the maintenance of postmitotic, long-lived neurons throughout adult life. The Bcl-2 family is comprised of anti-apoptotic members such as Bcl-2, Bcl-X_L, Bcl-w, Mcl-1, Bfl-1, Bcl-B, and pro-apoptotic proteins which include Bax, Bak, Bad, Bok, Bik, Bid, Bim, Hrk, Blk, Bnip3, Noxa, Puma, and Bcl-G. The sequence homology among Bcl-2 family proteins is confined to four Bcl-2 homology (BH) domains. One of the features of Bcl-2 family is the formation of homo- and heterodimers whose relative abundance coincides with either cell death or survival.³

Bcl-2 has been shown to rescue various neuronal populations from cell death due to neurotrophic factor deprivation,⁴ axotomy⁵ or other insults.⁶ Consistent with its apparent role in controlling developmental cell death, Bcl-2 is expressed at high levels in the developing central nervous system and eye.^{7–12} However, Bcl-2 levels decline rapidly during development in all except a few CNS neuronal populations, remaining at relatively high levels primarily in the peripheral nervous system.¹³

The Bcl-2 homolog, Bcl-X, is alternatively spliced into Bcl-X_L and Bcl-X_S, but only Bcl-X_L is expressed in the mouse CNS.^{14,15} In contrast to Bcl-2, high levels of Bcl-X_L are maintained throughout development into adulthood.^{13,16–19} Although Bcl-X_L can bind to Bax, its preferred heterodimerization partner appears to be Bak, another Bcl-2 family protein that generally functions to facilitate cell death.^{20,21}

Bid, a 'BH3-only' death promoting member of the Bcl-2 family,²² can be cleaved by caspase-8 following Fas/TNF-R1 activation.^{23,24} The p15 cleaved form of truncated Bid (tBid) translocates to mitochondria and induces release into the cytosol of cytochrome *c*, which binds to Apaf-1 and activates caspase-9 in the presence of dATP.²⁵ The activation of the initiator caspases, caspase-8 or caspase-9, leads to the activation of downstream effector caspases, such as caspase-3, -6, and -7, which cleave a number of cellular proteins, facilitating the destruction of the cell.²⁶ Thus, Bid represents an important mediator of cross-talk between the death receptor and mitochondria (caspase-9) pathways. Bid shares structural and functional similarity with other Bcl-2 family proteins known to have channel-forming activity, but its channel activity is dependent on proteolytic cleavage.²⁷ Interestingly, though capable of forming ion channels, the ability of Bid to induce cytochrome *c* release has been suggested to be mediated by heterodimerization with Bax. In this regard, Bid can facilitate the insertion of Bax into mitochondrial membranes to form functional oligomers.²⁸ Similarly, activated tBid has been reported to cause an allosteric activation of Bak,

inducing its intramembranous oligomerization and creating pores for cytochrome *c* efflux.²⁹

The physiological importance of these molecules for neuronal survival has been demonstrated by gene targeting. Six Bcl-2 family gene knockouts have been described,³⁰ Bcl-2, Bcl-X_L, Bcl-w, Bax, Bid, and Bim. No gross defects were apparent in the central and peripheral nervous system in Bcl-2 knockout mice.^{31,32} The Bcl-X_L-/- mice showed a severe phenotype, with the animals dying at embryonic day 13. The mice exhibited massive cell death of postmitotic immature neurons in the brain, spinal cord, and dorsal root ganglia.³³ Deletion of the Bax gene has an opposite effect: in both sympathetic and motor neuron populations, cell numbers in newborn mice are increased. Neonatal sympathetic neurons and facial motor neurons from Bax-deficient mice survive Nerve Growth Factor deprivation and disconnection from their targets by axotomy, respectively.³⁴ Bid-/- mice have no apparent developmental abnormalities in their nervous system, and have normal brains with respect to weight and morphology.³⁵

The aim of the present study was to provide a comprehensive overview of the developmental expression of several members of the Bcl-2 family (Bcl-2, Bcl-X_{L/S}, Bax, Bak and Bid), thus gaining a better understanding of how differences in expression of Bcl-2 family proteins may contribute to the control of brain development. Our observations provide the first insights into the *in vivo* expression of Bid and Bak proteins in the development of the murine nervous system.

Results

For clarity, we present our results contrasting the dynamics of Bid expression with the immunoreactivity of Bcl-2, Bax, Bcl-X and Bak in the developing and adult murine nervous system. The following major developmental stages are reported separately below: Early embryonic development (E4.5–8/TS6–12); Early neural development (E8.5–11.5/TS13–19); Early fetal life (E12–14.5/TS20–23); Later fetal life (E15.5–17.5/TS24–26); and Postnatal life and adulthood (P0 to 3 months).

Early embryonic development (E4.5–8/TS6–12): two and three-layered germ disc

At the earliest age examined (E6.5/TS9), Bid was strongly expressed in most cells throughout both the endo- and ectoderm (Figure 1A). At the onset of organogenesis and neurulation (E7.5–8/TS11–12), the predominant Bid expression was observed in the primitive neural tube and brain vesicles and in the caudal part of neural tube that differentiates into the spinal cord (Figure 1B). The pattern of labeling for Bcl-2 and Bax differed from that for Bcl-X_L and Bak (Figure 2 (Part 1A–D)). The Bcl-2 and Bax immunostaining occurred with similar distribution patterns as that seen for Bid (Figures 1A and 2 (Part 1A,C)). The immunoreactivity took the form of intense, coarse granular staining of the cytoplasm. In contrast, Bcl-X_L immunoreactivity was present in only trace quantities and Bak expression was confined to fine granular

labeling of the ectoderm and outer endoderm (Figure 2 (Part 1B,D)). In slightly older embryos (three-layered germ disc), Bcl-X_L expression began to appear in the apices of ectodermal cells, while Bak immunostaining remained faint in the endoderm.

Early neural development (E8.5–11.5/TS13–19)

In the early neural plate and neural tube stages (E8.5 to E9.5/TS13–15), all Bcl-2 family proteins examined remained strongly expressed predominantly at the pial and ventricular surfaces of the primitive brain vesicles (Figures 1B and 2 (Part 2A–E)). All markers exhibited intense coarse, granular, perinuclear immunostaining of the cytosolic region of cells.

The E10.5–11.5 (TS17–19) stage was characterized by a very high level of BID expression in the developing central and peripheral nervous system, including cranial and dorsal root ganglia as well as peripheral nerves (Figure 1C). This axonal immunoreactivity of Bid decreases at the later stages and becomes restricted to the neuronal perykarya, both in the central and peripheral nervous system. Although the proliferating population of neuroblasts, surrounding the ventricles in concentric layers, showed moderate positive staining, an obvious gradient of Bid expression appeared at this stage, associated with differentiation of the peripherally migrating progeny of ventricular neuroblasts. The cells with early features of neuronal differentiation in the mantle layer which gives rise to the gray matter of the basal ganglia, brainstem nuclei, cerebellum and spinal cord demonstrated strong cytosolic Bid immunostaining.

The first significant shift in the pattern of Bcl-2, Bax, Bcl-X and Bak labeling became apparent at E10.5 to E11.5/TS17–19 (compare Figures 2 (Part 2–4) and 3 (Part 1)), shortly after closure of the posterior neuropore at E9. At E10–11.5/TS16–19, two important features of Bcl-2 immunoreactivity became apparent. For the first time, a generalized cytoplasmic expression of Bcl-2 was evident in cells (Figure 2 (Part 2F)). This shift in immunolocalization is suggestive of a change in the subcellular distribution of the Bcl-2 protein. Second, Bcl-2 staining strengthened in migrating postmitotic neurons, particularly in the rhombencephalon (Figure 2 (Part 3A)), along the developing striatum (Figure 2 (Part 4A)), visceromotor column, and dorsal root ganglia (Figure 3 (Part 1A)). Denser and more compact Bcl-2 immuno-reactivity also began to appear in the somata and in the elongated processes of postmitotic neurons approaching their settling sites near the pial surface (Figure 2 (Part 2F)).

In contrast, Bax became more restricted compared to previous stages. Bax immunoreactivity began to decline in the neuroepithelium at E9.5–E10/TS15–16 (Figure 2 (Parts 2C, 3C, 4C)), but the labeling was still concentrated along the cell membranes of migrating neurons and it appeared in increasing concentrations over the following days in the cytoplasm of settling neurons, particularly in the ventral horn and isolated parts of rostral CNS (Figure 3 (Part 1C)).

For the first time, a significant increase of Bcl-X_L immunoreactivity began to appear in postmitotic neurons,

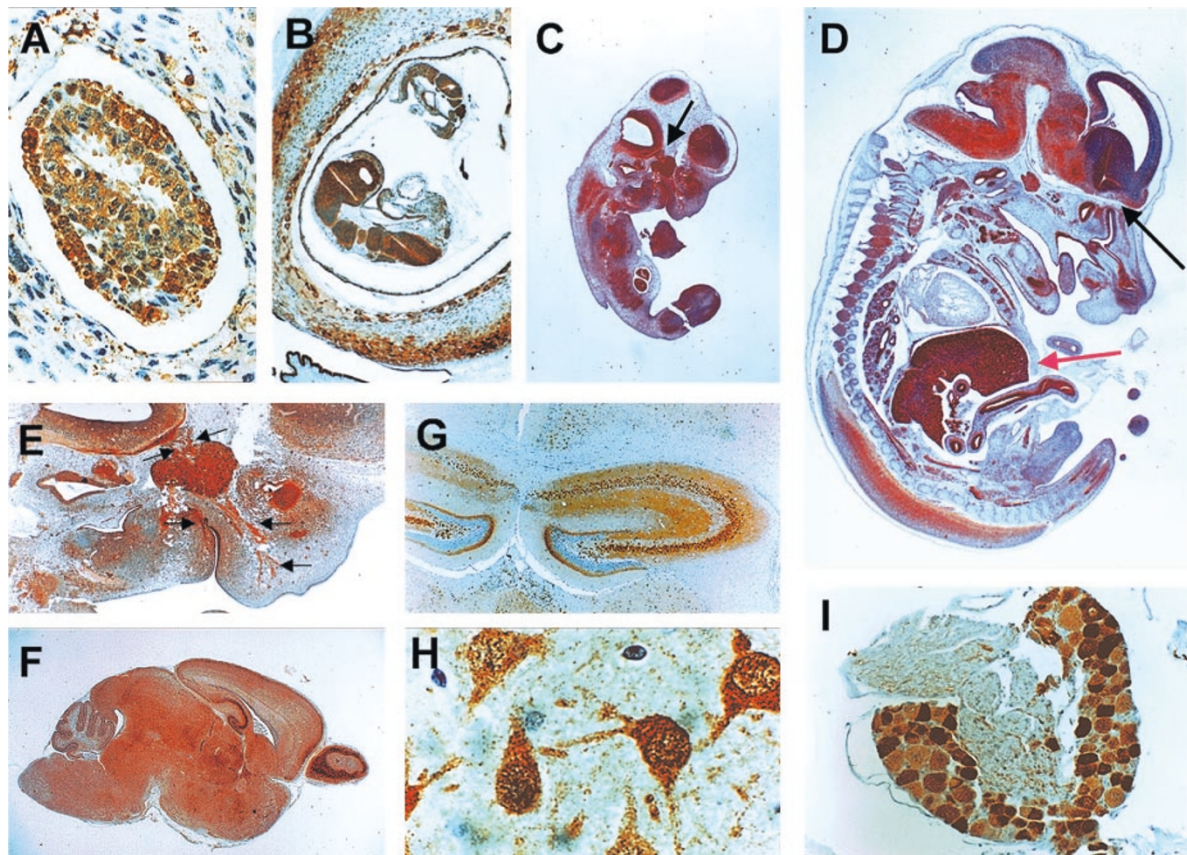


Figure 1 Bid expression in the developing nervous system. (A) E6.5/T9: early embryonic development. Transection of the embryonic disc shows diffuse Bid expression in the most embryonic cells. Note the presence of Bid in the subjacent hypoblast (a layer of endoderm), and in a superior ectodermal epiblast layer, which gives origin to the neural and surface ectoderm. (B) E7.5–8/T11–12. At the onset of organogenesis and neurulation, Bid immunostaining is most prominent in the primitive neural tube or in primitive brain vesicles and in the caudal part of neural tube differentiating into the spinal cord. (C) E10.5–11/T17–18: early neural development. These stages of early neural development were characterized by a very high level of Bid expression in the developing central and peripheral nervous system, including cranial and dorsal root ganglia, as well as peripheral nerves. The arrow indicates developing semilunar ganglion and nerve fibers of trigeminal cranial nerve V, demonstrating very strong Bid immunoreactivity. The axonal Bid expression was transient and not seen in the later stages after E14.5/T23. (D) E14.5/T23: early fetal life. During early fetal life period, intense Bid immunostaining appears in neurons of the emerging thalamus, hypothalamus, and in the brainstem in reticular formation, all neuronal centers involved in visual signal transmission, and major nuclei of the cranial nerves (V–XII). Bid is present not only in the primary olfactory neurons located in the olfactory epithelium, but also in their projection neurons and their synapses in the glomeruli in the forming olfactory bulb (long arrow). Apart from the nervous system, mainly liver (thick arrow) pluripotential and multipotential cells giving the origin to monocytic and granulocytic lineages were among the highest expressers of Bid. In addition, endodermal epithelia of digestive tract, bronchi, kidney, pancreas, salivary glands and gonads (oocytes or spermatogonia) as well as lymphoid organs, particularly the thymus, showed moderate to strong Bid immunostaining during this and further stages of intrauterine organogenesis and in the postnatal mouse development (not shown). (E) E12/T20. Trigeminal semilunar ganglion cells (refer to Part C) and their axons sprouting towards mesencephalic nucleus of trigeminal nerve, contain high levels of Bid expression (arrows). The labeling of nerve fibres was only transiently positive in the early stages but was not observed postnatally. (F) Postnatal day 7 (P7). Strong Bid immunostaining is observed in the brain of a 7-day-old pup, and continues into adulthood. In the sagittal sections, the highest level of Bid expression is seen in the limbic system, starting from the intense staining of mitral cell layer in the olfactory bulb, hippocampal sectors CA1–CA4, dentate gyrus, and associated centers in the basal ganglia including amygdala. Also in the cerebellum, high Bid content in the Purkinje cell layer is observed. (G,H) Adult brain hippocampus. Within the hippocampus, Bid is present at the highest levels in the CA3–CA4 neurons (G), particularly large hilar neurons (H), but is absent in most granular neurons of the CA1 region. The other neurons in the cortex and basal ganglia show moderate labeling for Bid protein. (I) Dorsal root ganglion of adult mice. In the peripheral nervous system, the ganglion cells located in the spinal and autonomic ganglia are among the strongest expressers of Bid. During E12/T20 to adulthood, the ventral motor columns of the spinal cord exhibit more Bid immunoreactivity than the dorsal column of sensory neurons (D) and Fig. 3 (Part 1F)

especially in the developing brainstem and spinal cord, particularly in the visceromotor column, ventral horn area, cranial and spinal nerve, dorsal roots and sensory ganglia (Figures 2 (Part 2B,3B) and 3 (Part 1B)). The elevation of Bcl-X_L levels in the CNS was accompanied by a strong Bid immunoreactivity in the same population of differentiating neurons. In the PNS, a high level of Bid protein and low Bcl-X_L expression were observed (Figure 3 (Part 1B,F);

DRG). A striking contrast in intensity of Bcl-2 versus Bcl-X_L immunostaining was evident at this time, particularly comparing proliferative, periventricular matrix zone, and differentiating cell compartments where inverse Bcl-2 and Bcl-X_L expression patterns were observed (Figure 2 (Part 2A,B)). Interestingly, however, Bcl-X_S was similar to Bcl-2 staining at this stage (compare Figure 2 (Part 2A and 2E)). In the next embryonic stages, Bcl-X_S expression was

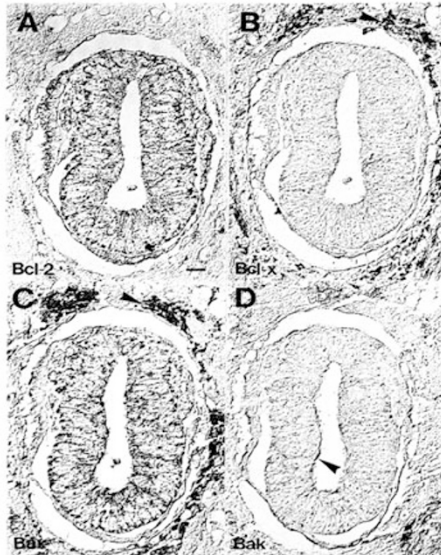
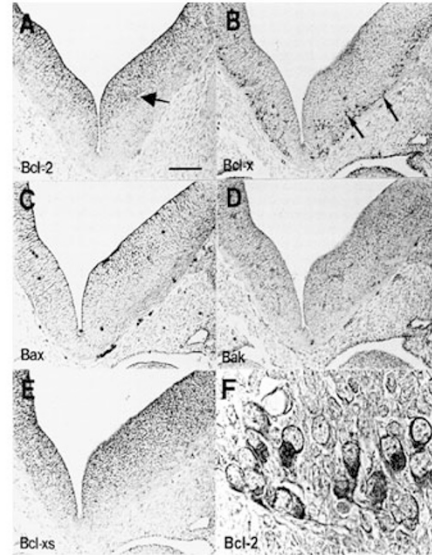
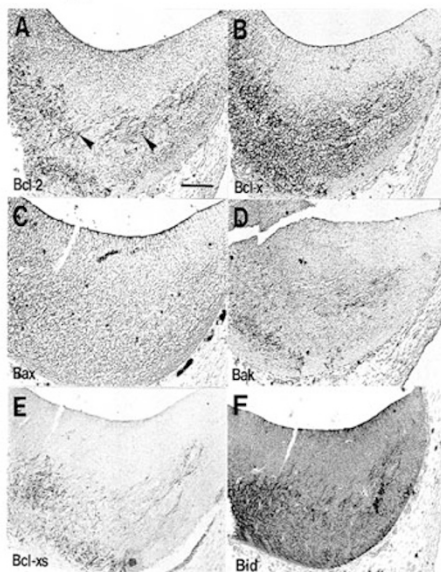
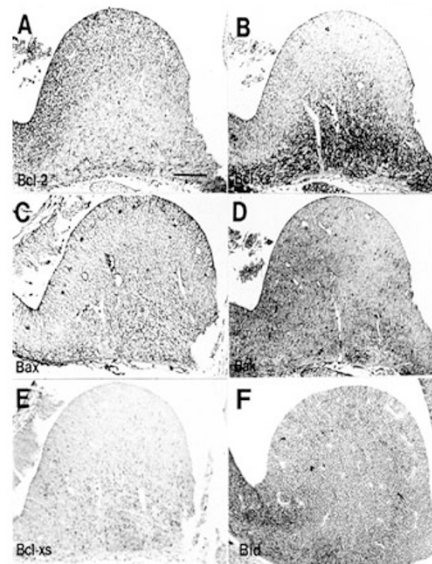
Fig. 2.1 E6.5/TS9**Fig. 2.2 E10.5/TS17****Fig. 2.3 E11/TS18****Fig. 2.4 E11/TS18**

Figure 2 Bcl-2 family proteins in early embryonic neural development of brain (E6.5/TS9 to E11/TS18). **Part 1A–D.** Transection of the embryonic disc at E6.5/TS9. (A) Note the dense granular Bcl-2 staining in both ecto- and endoderm. (B) Bcl-x immunoreactivity is found only in maternal blood cells (arrowhead). (C) Bax immunoreactivity is predominantly in embryonic ectoderm (arrowhead). (D) Bak is represented by only a few immunoreactive granules along the inner margin of the embryonic ectoderm (arrowhead). (Scale bar indicates 10 μ m for all photomicrographs.) **Part 2A–F** Bcl-2 family immunoreactivity at E10.5/TS17 shows moderate to intense fine granular labeling throughout the proliferative region of the posterior rhombencephalon (A–D; transverse section). Bcl-X_L expression is moderate in the proliferating cells, but high in migratory, postmitotic neurons (B; arrows). Bcl-X_S expression is found for the first time in the form of the coarse granules mainly in some cells of the proliferative zone. (F) Shows a representative example of cytoplasmic, diffuse Bcl-2 expression in differentiating motoneurons of hypoglossal nucleus, indicated by arrow in A. Scale bar indicates 100 μ m for all photomicrographs. **Part 3A–F** (E11/TS18). (A) Sagittal section through the pons at E11/TS18, shows Bcl-2 immunopositive cells (arrowheads) migrating from the proliferative zone. (B) In the adjacent section, Bcl-X_L immunoreactivity is widespread in all cells outside the proliferative compartment. (C) Bax immunoreactivity is strong along the ventricular surface, but weak in the differentiating neurons. (D) Occasional differentiating neurons express Bak immunoreactivity. (E) Bcl-X_S staining shows a pattern similar to Bcl-2, but the proliferative compartment does not contain any Bcl-X_S expression. (F) Similarly to Bcl-X_L, Bid expression was moderately high in most cells, but particularly higher in postmitotic differentiated neurons in neuronal centers of the tegmentum. Scale bar indicates 100 μ m for all photomicrographs. **Part 4A–F** Bcl-2 family immunoreactivity in adjacent sections through the developing striatum at E11/TS18. (A) Bcl-2 immunoreactivity is concentrated in the proliferative compartment adjacent to the ventricular surface. (B) Inversely, Bcl-X_L becomes strongly expressed in the differentiating cells of the early striatum at E11/TS18. (C) Bax immunoreactivity is present along the ventricular surface and in the adjacent parts of the proliferative compartment. (D) Bak immunoreactivity is poorly and diffusely expressed in the striatum. (E) Bcl-X_S is localized faintly to the differentiating neurons, but not expressed in the cells of the proliferative compartment. (F) Uniformly distributed Bid immunostaining is seen in undifferentiated cells in developing striatum. Scale bar indicates 100 μ m for all photomicrographs

confined only to selected postmitotic neurons in Bcl2/ Bcl-X_L positive areas, declining in early fetal life.

Bak immunoreactivity at this age (E11/TS18) began to increase in a focal manner relative to the ventricular zone, with slight elevation in postmitotic areas where the neurons already exhibited strong positivity for Bcl-2 and Bcl-X_L. Bak immunoreactivity continued to increase in intensity compared to previous stages, but still remained relatively weak (Figures 2 (Part 3D,4D) and 3 (Part 1D)). Moderate intensity of Bak immunostaining was apparent in the region of the visceromotor complex of the myelencephalon (putative solitary nucleus), with only occasional crescent-shaped cellular segments labeled in the spinal cord and isthmus.

TUNEL positivity was found only in occasional scattered nuclei or apoptotic bodies in both proliferative and postmitotic areas of CNS, in contrast to the rich TUNEL activity seen in the forming eyelids, ear, nose and interdigital areas of the upper and lower extremities, and liver (not shown). There was no particular correlation to expression of any of the Bcl-2 family proteins.

Early fetal life (E12–14.5/TS20–23)

The E12/TS20 stage of development was characterized by increased restriction of the Bcl-2 family proteins to neurons, particularly in the most differentiated neurons of the reticular formation in the brainstem and major nuclei of the cranial nerves (V–XII) and motoneuron column. By E12.5/TS21, Bid was strongly expressed in the region of the forebrain, which later develops to olfactory lobe, but it was barely present in the olfactory epithelium and numerous bundles of non-myelinated neurons which constitute the olfactory nerves. However, not until E14.5 (TS23) is the olfactory bulbs formed, demonstrating very high levels of Bid immunostaining, mainly in the differentiating mitral cells (Figure 1D). Thus, Bid is present not only in the primary olfactory neurons located in the olfactory epithelium, but also in their projections to the glomeruli in the olfactory bulb. The glomeruli (comprised of the terminals of the olfactory receptor cells and the dendrites of mitral, tufted, and periglomerular cells) contain strong Bid staining. The cell bodies of granule neurons were negative throughout all stages. Neurons in amygdala, which are the main recipient of the projections from the olfactory bulb, also expressed high levels of Bid.

With progression of fetal life (E12 to E14.5/TS21–23), the immunoreactivity of Bcl-2, Bax, Bcl-X_L and Bak was overall low, except for restricted populations of neuronal cells (Figure 3 (Part 2A); arrowheads). The Bcl-2 expression took the form of a gradient, with high protein content in differentiated neurons located caudally and low Bcl-2 levels in the rostral centers (striatum, neocortex). High immunoreactivity areas included dorsal root ganglion cells, dorsal horn of the spinal cord (highest levels at E13.5 to E14.5/TS22–23), the somatomotor column (particularly cranial nerve nuclei), superficially located neurons of lateral tegmentum in the brainstem, and habenular nuclei (Figure 3 (Part 2A)). The neocortex and differentiating basal ganglia contained weakly positive Bcl-2 neurons (Figure 3 (Part 2A,4A)), as opposed to the rising levels of the

widespread Bcl-X_L labeling. At E13.5–14.5/TS22–23, very strong Bcl-X_L immunoreactivity was noted in the differentiating neurons of the dorsal thalamus, hippocampal cortical plate, other allocortical areas, and particularly in the brainstem (Figures 3 (Part 2B,3A) and 4 (Part 1A)).

Parallel to Bcl-X_L, Bax immunoreactivity continued to increase through the E12–14.5/TS20–23 period. The labeling was relatively poor in the ventricular zone, but intense perisomatic, neuropilar staining was noted around differentiating neurons, particularly in the hippocampal allocortex and thalamus (Figure 3 (Part 2C)). The Bak staining pattern was unique and did not overlap the expression of any other marker (Figures 3 (Part 2D,3B) and 4 (Part 1B,C)). Bak expression became concentrated in the midline of caudally located parts of the developing central nervous system (hindbrain and spinal cord), both in glial structures of the raphe and in differentiating neurons of paramedian nuclei in the brainstem (Figure 4 (Part 1B,C)).

Later fetal life (E15.5–17.5/TS24–26)

The fetal period from E15.5–17.5/TS24–26 was characterized by a decrease of Bcl-2 expression, accompanied by a parallel increase of Bcl-X_L. High levels of Bid, and Bax were also present in the developing brain, but not Bak (Figures 1D and 3 (Part 4C,D)). No Bcl-X_S immunoreactivity was found during later fetal life (E15.5–17.5).

During E14.5–16.5 (TS23–25), the enlarging basal ganglia express higher levels of Bid (Figure 1D). At this time, the thalamus and hypothalamus increase in size and corpus striatum differentiates into caudate and lentiform nucleus, respectively, with the latter subdividing into the putamen and the globus pallidus or pallidum. A high level of Bid expression was observed in many neurons within these structures, particularly in the large size ones (Figure 1D). The neuronal centers involved in the visual pathway, such as retinal ganglion cells and lateral geniculate nuclei and many others in the brainstem, were highly immunoreactive for Bid. Neurons in the medial geniculate nucleus and inferior colliculus in the auditory pathway contain moderate Bid expression.

During the E15.5 to E17.5/TS24–26 stage (Figures 3 (Part 4/4A) and 4 (Part 2/2A)), Bcl-2 expression continued to decline, and the labeling that was present was concentrated in discrete groups of differentiating neurons. Immunoreactivity of the ventricular zone was minimal except for some fine granular labeling along the course of the olfactory migration. Postmitotic scattered neurons expressing Bcl-2 at this age included the substantia nigra of the midbrain, the cerebellar or deep nuclei, the dorsal thalamus and dorsal/ventral striatum, hypothalamus and septal nuclei, amygdala and entorhinal cortex neurons, as well as subplate and upper cortical plate neurons in the isocortex (Figures 3 (Part 4A) and 4 (Part 2A)). Within the cortex, the upper cortical plate was also immunoreactive for Bcl-X_L (Figures 3 (Part 2B) and 4 (Part 2B)), as were apparently migrating neurons in the intermediate zone. By this age, Bax expression began to increase in ventricular and subventricular zones of the cortex and striatum (Figure

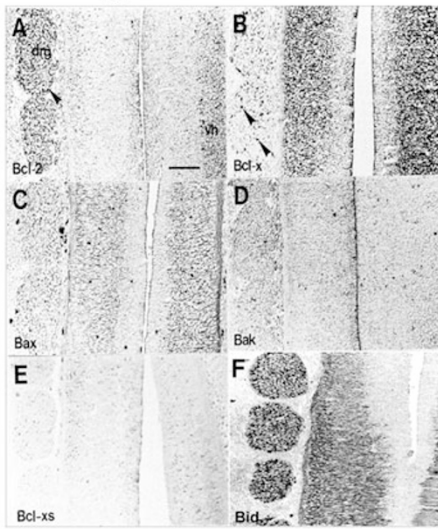
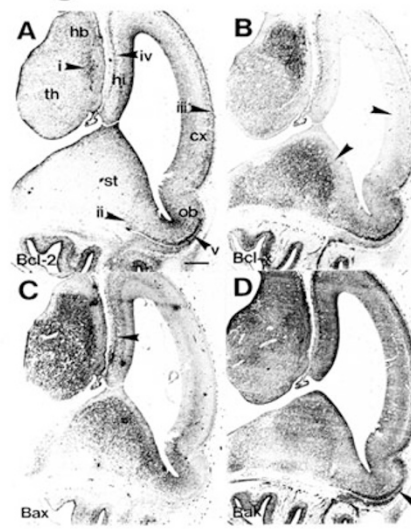
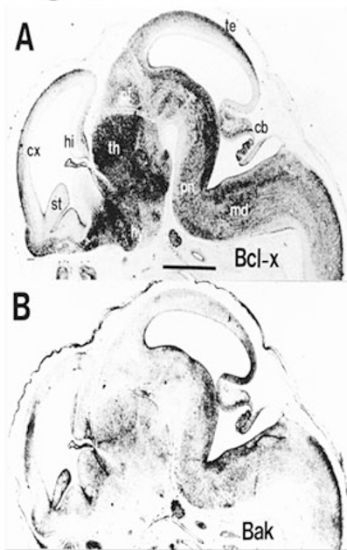
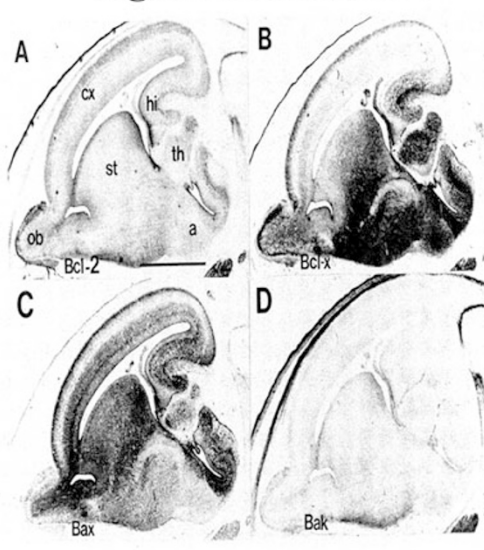
Fig. 3.1 E11/TS18**Fig. 3.2 E13.5/TS22****Fig. 3.3 E14.5/TS23****Fig. 3.4 E15.5/TS24**

Figure 3 Bcl-2 family protein expression in embryonic neural development at E11–E15.5/TS18–24. **Part 1A–F:** Expression of Bcl-2 family genes in spinal cord and DRGs: Longitudinal sections through the thoracic spinal cord and dorsal root ganglia at E11/TS18. (A) Bcl-2 immunoreactivity is finely granular within the neuroepithelium along the ventricular surface, stronger within the ventral horn (vh) and even more pronounced in the dorsal root ganglia (drg, arrowhead). (B) Bcl-X_L is strongly expressed in the differentiating neurons of the ventral horn, and occasional dorsal root ganglion cells (arrowheads). (C) Bax immunoreactivity is found in a perisomatic location in the outer proliferative compartment and in the ventral horn cells, as well as DRG cells. (D) Bak immunoreactivity is localized to the ventricular surface of the midline and in occasional DRG cells. (E) Bcl-X_S immunopositivity is found in the inner neuroepithelium, and among selected ventral horn and DRG cells. (F) Along with Bcl-2 and Bcl-X_L, Bid immunoreactivity was the strongest in the ganglion cells of DRG, and in the ventral and dorsal column of the spinal cord at this stage of development. Scale bar indicates 100 μ m. **Part 2:** Sagittal sections through a fetus at E13.5/TS22 showing cortex (cx), habenula (hb), hippocampus (hi), olfactory bulbs (ob), striatum (st), thalamus (th). (A) Bcl-2 immunoreactivity is moderately strong in the thalamus, with concentrations in the anterior region of the habenula (arrowhead i). In the striatum, Bcl-2 is present at moderate levels only along the ventricular zone, with single scattered immunoreactive neurons in the ventral striatum and olfactory tubercle (arrowhead ii). In the cortex, more intense labeling is seen in the cortical plate, particularly in the upper zone (arrowhead iii). In the hippocampus, Bcl-2 immunoreactivity is strong in the developing pyramidal layer (arrowhead iv). In both the cortex and hippocampus the labeling with Bcl-2 is lower in the intermediate zone than above or below. In the olfactory bulb, Bcl-2 immunoreactivity is strong in the mitral cell layer (arrowhead v) of the main, but not accessory, olfactory bulb. (B) Bcl-X_L shows positive cells throughout the habenula and differentiating striatal and substriatal tissue. Like Bcl-2, it is predominantly in the upper layers of iso- and allocortical areas, but proliferative zones, which are positive for Bcl-2, are negative for Bcl-x (arrowheads in cortex and striatum). (C) Bax is very high in the differentiating thalamus and subventricular zones of both the striatum and olfactory bulb, but absent from the differentiating area of isocortex and olfactory bulb. Bax immunoreactivity is strong in the developing pyramidal layer of the hippocampus (arrowhead). (D) Bak immunoreactivity is widespread, but strongest in the mitral cell layer of the olfactory bulb (arrowhead). Scale bar indicates 100 μ m. **Part 3:** Bcl-x and Bak immunoreactivity in closely adjacent sagittal sections (A, B) at E14.5/TS23. Bcl-X_L immunoreactivity (A) was particularly strong in the thalamus (th), hypothalamus (hy), ventral striatum, superficial tectum (te), pons (pn) and medulla (md). An association with developing nuclei is evident. Bak immunoreactivity (B)

3 (Part 4C)), and was particularly high in the allocortex, in the same areas which also exhibited strong Bcl-X_L staining. Bax labeling was also strong in the neuropil of the upper cortical plate and in migrating neurons of the intermediate zone of the cortex (Figure 4 (Part 2C)).

In comparison to stage E15.5/TS24, at this late stage of fetal life (E17.5/TS26), Bak immunoreactivity (both neuronal and neuropilar) became stronger in the midline neuronal centers. This immunostaining was relatively intense compared to the other Bcl-2 family members. A particularly

Fig. 4.1 E14.5/TS23

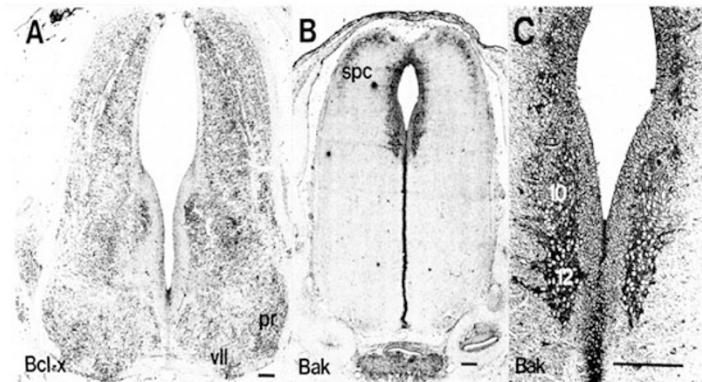


Fig. 4.2 E15.5/TS24

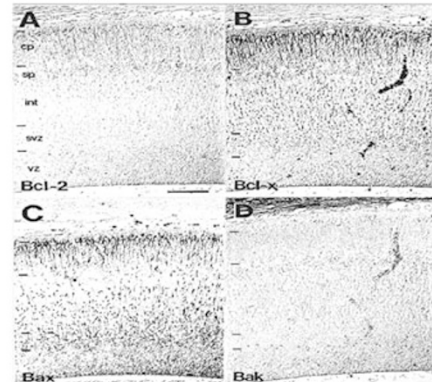


Fig. 4.3 E18.5/P0

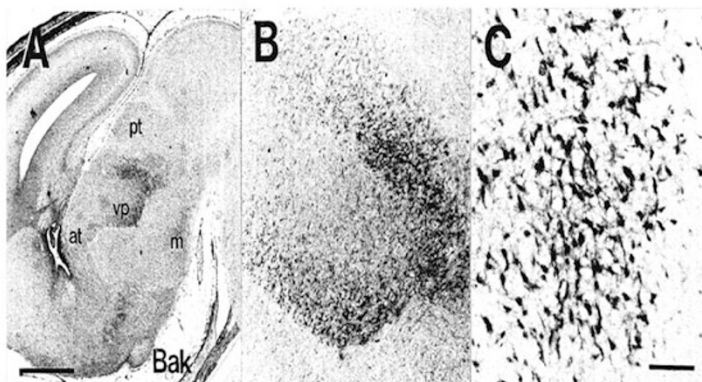


Fig. 4.4 E18.5/P0

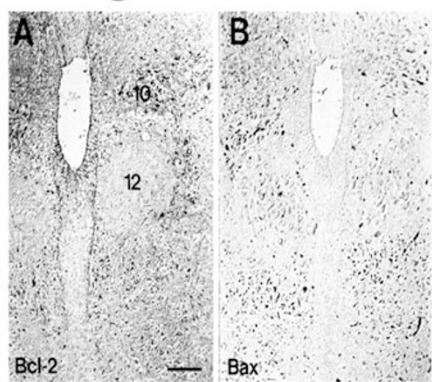


Figure 4 Bcl-2 family protein expression in the brainstem, cortex, and thalamus at E14.5/TS23 to birth. **Part 1** Bcl-x and Bak immunoreactivity in the brainstem at E14.5/TS23. **(A)** Bcl-X_L is strongly expressed in the differentiating cells of the pons and medulla. Principal trigeminal nucleus (pr); ventral nucleus of the lateral lemniscus (vll). **(B, C)** Low and high power views of Bak immunoreactivity in the pons and medulla, showing strong expression in the midline glia, upper layers of the caudal subnucleus of the trigeminal spinal tract (spc) and cranial nerve nuclei: 10=dorsal motor nucleus of the vagus, 12=hypoglossal nucleus. Scale bars indicate 100 μm. **Part 2** Sections through the parietal cortex at E15.5/TS23, showing strong immunoreactivity for Bcl-X_L and Bax in the upper layers of the cortical plate (cp) as well as the intermediate zone (int). Bax immunoreactivity is also apparent in the ventricular zone (vz) and subventricular zone (svz). Bak immunoreactivity is poor in the cortex, while Bcl-2 immunoreactivity is only weakly present in the cortical plate and subplate (sp). Scale bar indicates 100 μm. **Part 3** Bak immunoreactivity in a sagittally sectioned E18.5/P0 animal, showing strong immunoreactivity in the thalamus: **(A)** anterior thalamic nucleus; mammillary nuclei (m); pretectum (pt); ventral posterior thalamic nucleus (vp). **(B, C)** Higher power views of the same section showing neuronal labeling in the ventral posterior thalamic nucleus. Scale bars indicate 1 mm **(A)** and 100 μm **(C)**. **Part 4** (E18.5/P0). Bcl-2 **(A)** and Bax **(B)** immunoreactivity in the brainstem at E18.5/P0, showing strong differential staining in the paramedian region: 10=dorsal motor nucleus of the vagus; 12=hypoglossal nucleus. Scale bar indicates 100 μm

was less intense, but also concentrated in the diencephalon and dorsal brainstem. Cerebellum (cb); cortex (cx); hippocampus (hi); pretectum (pt); striatum (st). Scale bar indicates 1 mm. **Part 4** Closely neighboring sagittal sections through the forebrain of an E15.5/TS24 fetus. In the isocortex (cx), Bcl-2 **(A)** is concentrated in the cortical plate and to a lesser extent in the proliferative zone. **(B)** Bcl-X_L isocortical labeling is found only in the superficial layers of the cortical plate. Bax **(C)** is colocalized with Bcl-X_L in the superficial cortical layers, but in addition is found in the intermediate and proliferative regions. Bak **(D)** is found only in trace amounts in the cortex. In the olfactory bulb (ob) on Part A-D, positive labeling for Bcl-2 is found in the olfactory nerve layer, Bcl-X_L is found predominantly in the mitral layer, but moderate labeling was found in the deeper layers of the bulb. Bax is found predominantly in the central proliferative areas close to the olfactory ventricle. Bak is found only in traces in the olfactory nerve fibers and mitral cell layers. In the hippocampus (hi), Bcl-2 is highest in granule cells of the dentate gyrus. In the striatum (st), Bcl-2 is found in the proliferative areas and differentiating ventrobasal forebrain. Bcl-x is present only in small amounts in the ventricular and subventricular zones. It is high to very high in the dorsal striatum, pallidum and ventral prosencephalon including the amygdaloid area (a). Bax is highest in the striatum, with only moderate levels in the ventral areas and low levels of immunoreactivity in the amygdaloid area. The highest immunoreactivity for Bak is found in the basal forebrain area including olfactory tubercle and in the striatal and olfactory bulb neuroepithelium. Scale bar indicates 1 mm

striking feature of Bak immunoreactivity at this age was labeling in a crescent shaped and precisely demarcated region of the dorsal thalamus, presumably corresponding to sensory thalamic nuclei such as the ventral posterior thalamic nucleus (Figure 4 (Part 3A–C)). Midline Bak immunoreactivity remained strong throughout the rostro-caudal extent of the CNS, particularly in motor nuclei of the brainstem (Figure 4 (Part 1B,C)). Labeling was also strong in the olfactory migratory stream, dorsal root ganglia and in the spinal cord (stronger in the ventral than dorsal horn), but weak in the cortex (Figure 4 (Part 2D)).

Postnatal life and adulthood (P0 to 3 months)

Towards the end of fetal life (E17.5–18.5/P0), a widespread reduction in Bcl-2, Bax and Bak immunoreactivity was evident. Expression of Bcl-X_L, although maintained at a rather high level, was confined mostly to differentiating cells. High expression of Bid in the brain at the late stages of embryonic development (E17.5/T26) continued to persist into adulthood. A striking example of this staining pattern is demonstrated in Figure 1F in a sagittal section of the whole brain, at seven days after birth (P7). Extremely high levels of Bid protein were seen in the limbic system, particularly in the mitral cell layer of the olfactory bulb, extending to the hippocampal sectors CA1–CA4, to nuclear centers in the basal ganglia, tectum mesencephali, and Purkinje cells in the cerebellum (Figure 1F). Similar to the granule neurons in the olfactory bulb, the dentate granular neurons in hippocampus and the cerebellar granular layer contained greatly reduced Bid staining (Figure 1G). Thus, large neurons in CA4 hippocampal hilus region (Figure 1H), mitral cells in the olfactory bulb, and Purkinje cells in the cerebellum, contained the highest levels of Bid protein (Figure 1F–H). The high expression of Bid in the ventral motor columns of the spinal cord, and in the spinal (Figure 1I) or autonomic ganglia in the peripheral system, persisted into adulthood. However, the high immunostaining intensity for Bid in the dorsal column of sensory neurons subsided postnatally.

The expression of Bcl-2, Bax, and Bak in postnatal life was present throughout most of the brain, but at only moderate or low levels compared to the fetal period (example for Bcl-2/Bax; Figure 3 (Part 4A,B)). Bcl-X_L immunoreactivity was maintained in neurons and neuropil at a relatively high level, predominantly in Purkinje cells in the cerebellum, in cerebral cortical neurons, and in pyramidal neurons of the CA3 region of the hippocampus. The expression of Bak protein in the dorsal thalamus during late fetal and early postnatal life was lost by the end of the first postnatal week. Bcl-2 showed intense focal expression in the ventral tegmental area, Bergman glia of the cerebellum, and in some cerebellar Golgi cells, while moderate to strong Bcl-X_L expression was found in the ventral tegmental area, cerebellar glomeruli, deep nuclei, and Purkinje cells.

Immunoblotting results

Overall, the results of the immunoblot analysis for Bcl-2 protein family were consistent with the immunohistochemical data. Bcl-X_L protein was easily detectable in the developing

cerebrum from ME12 through adulthood, only slightly declining in the adult brain (Figure 5A). Bcl-X_L protein levels were increased in cerebellum and spinal cord at late embryonic stages and postnatally, compared to early fetal life. The appearance of two bands for Bcl-X_L is typical of its migration in SDS-PAGE experiments,¹⁵ and has recently been attributed to deamination of an asparagine residue in the protein.³⁶ Bcl-X_S was not found in any specimen tested at any stage, due probably to its very restricted expression during nervous system development.

Bcl-2 protein was detectable by immunoblotting in the cerebrum at the earliest embryonic time examined (ME12), and in the cerebellum as early as the ME16 stage, persisting into adulthood (Figure 5A). Only trace levels of Bcl-2 protein were observed by immunoblotting in the spinal cord at ME12 and ME13, but not in the adult mouse spinal cord.

Similarly, Bax protein levels in the cerebrum were highest at ME12, declining thereafter. Bax protein was also clearly detectable by immunoblotting in the embryonic cerebellum and spinal cord, but was not found in the corresponding adult tissues.

Finally, Bak protein was detected by immunoblotting in the cerebrum at ME13 through ME15, before declining to undetectable or barely detectable levels. In the cerebellum, Bak protein was not expressed at ME14 but appeared at ME16 and was maintained in adult tissues. In the developing spinal cord, Bak was first detectable by immunoblotting at ME13 and persisted into adulthood.

Bid immunoblotting data showed barely detectable levels in early embryonic stages (ME11–ME12) with the first immunodetectable Bid protein appearing at ME13–14. The high levels of Bid protein were detectable by immunoblotting in the adult cerebrum, cerebellum and spinal cord (Figure 5A). Thus, the Western blotting investigation confirmed the presence of high Bid levels in the adult cerebrum, cerebellum and spinal cord, in agreement with immunocytochemical observations. The inability to detect Bid by blotting at the earlier perinatal stages may result from the technical limitations of the immunoblotting method, caused by dilution of Bid-positive cells by non-expressing cells. A p15 fragment corresponding to cleaved Bid was observed in brain specimens derived from late fetal and postnatal stages, suggesting the likelihood of caspase-mediated cleavage of this protein during those times.

A summary of major developmental changes in Bcl-2 family expression in the developing CNS is presented in Figure 6, which depicts graphically the dynamic expression of Bcl-2, Bax, Bcl-X_L, Bak and Bid in the developing and adult CNS.

Discussion

This study characterizes for the first time the distribution of the Bid and Bak proteins in the developing and adult nervous system by immunohistochemistry and immunoblotting. Two different populations of cells express Bid in the developing nervous system, the proliferating stem cells of ventricular zones and postmitotic neurons. Interestingly, as differentia-

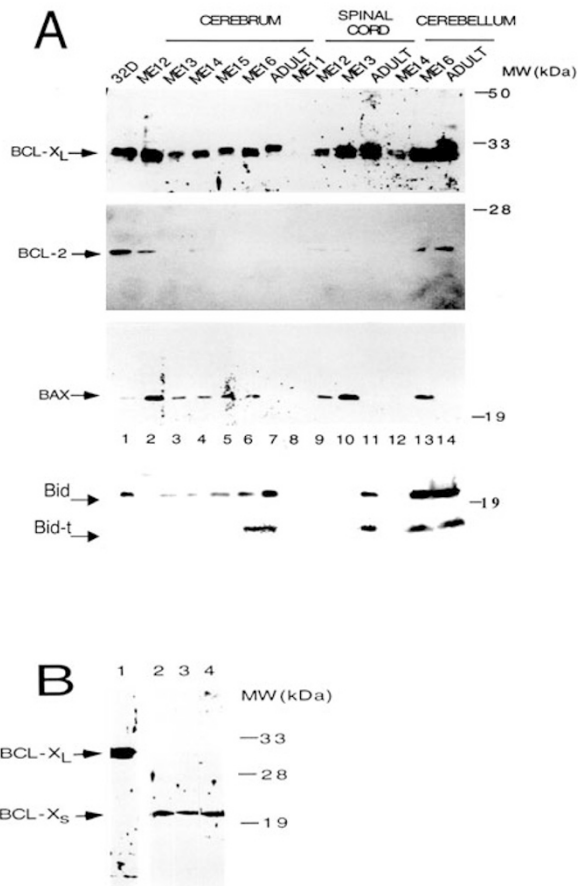


Figure 5 Immunoblot analysis of Bcl-2 family expression in developing brain. **(A)** Relative levels of Bcl-2 family proteins. The specimens from cerebrum included both cerebral hemispheres and basal ganglia in the early neural developmental stages (E12–13/TS21–22); forebrain cerebral cortex derived from ME14, 16 and adult mice. The spinal cord specimens from ME11, 12, 13, and adult, and cerebellar specimens from ME14, 16, and adult mice were investigated. 32D mouse T-cell leukemia cell line was used as a positive control (lane 1). Protein lysates (50 μg) were prepared in RIPA buffer, subjected to SDS-PAGE (12% gels) and transferred to nitrocellulose membrane. The membrane was reprobbed with rabbit monospecific antibodies for Bcl-X_L, mBcl2, mBax, and Bid **(A)**, with ECL-based detection using the multiple antigen detection (MAD) immunoblotting procedure.⁴⁸ Bcl-X_L protein was easily detectable in the developing cerebrum from E12/TS20 through adulthood, slightly declining in the adult brain **(A)**; upper panel). In the cerebellum and spinal cord, a marked up-regulation of Bcl-X_L protein at the late embryonic stage and adulthood as compared to early fetal life, inversely correlated with the dynamic of the Bcl-2 expression. The next panel demonstrates the dynamic of Bax expression, with the highest level at the early stages of CNS remodeling (E12/TS21 in cortex, E13/TS22 in the spinal cord and E16/TS25 in the cerebellum). Note the complete decrease of Bax production in the entire CNS during adulthood. Western blotting for Bid demonstrated a steady increase in the expression of this protein during neural development, with a barely detectable signal in the early embryonic stages (E11–12/TS18–20). The first discernible Bid expression was seen in the early fetal life (E13–14/TS22–23), and increased in the adult specimens. p15 fragment of cleaved Bid could be seen in the specimens from late fetal or postnatal stages, in particular in the cerebellar specimens. **(B)** Characterization of antibodies to long and short isoforms of Bcl-X. Jurkat cells were either untreated (lanes 1 and 2), treated with 10 j/m² UV radiation and then maintained for an additional 4 h in culture (lane 3) or incubated with 1 μg/ml anti-Fas mAb (CH-11) for 6 h. Cells were then lysed and protein samples (100 μg) were analyzed by SDS-PAGE immunoblotting. Bcl-X_L (lane 1 probed with 1695 antibody) and Bcl-X_S (lanes 2–4 probed with 1688 antibody) were detected using specific anti-sera

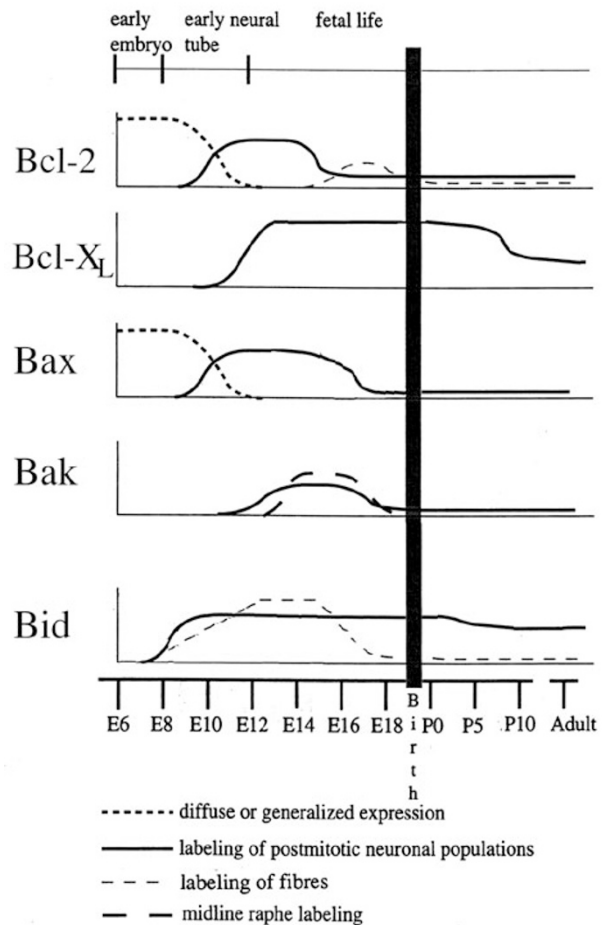


Figure 6 Schematic diagram summarizing the predominant changes in Bcl-2 family expression during embryonic and neural development. During early embryonic development, the predominant expression is of Bcl-2, Bax and Bid, whereas Bcl-X_L and Bak are almost absent. At E10–11/TS16–18, after formation of the neural tube, the general expression of Bcl-2 and Bax was down-regulated, only selected groups of differentiating neurons expressed high levels of these proteins. High level of expression in postmitotic neurons was the main feature of Bcl-X_L and Bid expression during early fetal life. Bcl-X_L was highest from E12 to E16/TS20–25. Bak immunoreactivity appeared slightly later compared to other family members, presenting a focal expression pattern. High expression of Bid persisted since the first appearance into adulthood and was mainly confined to the lower levels of CNS; e.g. spinal cord, brainstem, basal ganglia and limbic system

tion proceeds, the differentiating cells express higher levels of Bid than the stem cells of the ventricular zone. Although Bid protein is present in the most vital regions of brain, both in the embryonic and postnatal life, *bid*^{-/-} mice had no apparent developmental abnormalities in the nervous system.³⁵ These findings suggest that Bid may not be essential for programmed cell death in the developing nervous system. However, the presence of the p15 Bid fragment in differentiating brain formations during late stages of CNS development requires further investigation.

The high levels of Bid expression in the entire limbic system (particularly in the olfactory bulb and hippocampus, large neurons of the basal ganglia, and brainstem), Purkinje cells in the cerebellum and motoneurons of the spinal cord,

could implicate the involvement of Bid in the increased sensitivity of these neuronal subpopulations to pathological death stimuli (e.g. ischemia), supporting the pathoclise theory of Vogt.³⁷ Accordingly, we speculate that *bid*^{-/-} mice should be more resistant to focal and global ischemia. The lack of brain abnormalities in *bid* knockout mice, similarly as in *bax* gene ablation, may indicate the existence of alternative and redundant pathways to control apoptosis during nervous system development. A double *bid* and *bax* knockout might reveal roles for these two proteins in murine brain development.

During early embryonic development, Bcl-2, Bax and Bid were predominantly expressed, while Bcl-X and Bak were nearly absent. This early co-expression of Bcl-2 and Bax agrees with the concept that cell survival is controlled in the early embryo by a balance between anti-apoptotic and pro-apoptotic molecules. After formation of the neural tube, expression of Bcl-2 and Bax was reduced overall. In particular, Bcl-2 labeling of the proliferative regions of the CNS was greatly reduced. The down-regulation of Bcl-2 may coincide with the onset of differentiation and exhaustion of the proliferative zones in many parts of the CNS. A reduction in Bcl-2 levels conceivably could leave Bax (and Bid) unopposed and thus promote involution of proliferative zones by apoptosis. Cell death in the neural proliferative zones therefore may represent a balance between Bcl-2 and Bax/Bid levels. An alternative pathway triggering neuronal death might be activated by expression of Bcl-X_S at early stages in the absence of opposition by Bcl-X_L or Bcl-2. An elevation of Bcl-X_L expression may explain why the ventricular proliferative populations do not completely break down after down-regulation of Bcl-2 in early and late fetal stages. As fetal development progresses, Bcl-2, Bax and particularly Bid expression shift towards subgroups of differentiating neurons, becoming restricted to subpopulations of developing neurons. The increased expression levels of the additional apoptosis agonist Bid at the later stages of neuronal differentiation, could sensitize the cells to a variety of death stimuli in response to certain pathological situations.

Bak immunoreactivity appeared slightly later than the other three Bcl-2 family members did, and its initial expression pattern was characterized by focal distribution in groups of differentiating neurons. Thus, the participation of Bak in neuronal apoptosis seems to be more limited and confined to a few groups of specialized neurons.

Migrating neurons in the brainstem and cortex may need the protective effects of Bcl-2 during their movement from the proliferative areas to their settling sites where trophic factors become available for neuronal maintenance or where cell adhesion events mediated by integrins are modulated. Once the cortical neurons reach the cortical plate, we speculate that Bcl-X_L expression becomes the predominant protective mechanism. Ferrer and colleagues¹⁰ have reported high levels of Bcl-2 immunoreactivity in all the cerebral cortical layers during the first week of postnatal life, and other groups have reported that a wave of cell death appears in the cerebral cortex late in the first postnatal week,³⁸ coincident with the diminution of Bcl-2 immunoreactivity. The early embryonic lethal effect of Bcl-

X_L ablation could be assigned to the lack of pro-life support for this protein and massive death of postmitotic neurons in the most vital centers of the brainstem, including reticular formation (center for circulation and breathing) and cranial nerves, expressing the highest Bcl-X_L level at E12–13.5.

The death-promoting proteins Bax and Bak but not Bid are greatly diminished in the cortex by postnatal life. Labeling for Bax in the intermediate zone of cortical plate may indicate the initiation of apoptosis either in surplus migrating neurons or redundant oligodendroglia in the developing white matter.³⁹

Both Bcl-2 and Bcl-X_L showed immunoreactivity in the superficial layers of the olfactory bulb during late fetal development. By contrast, the olfactory bulb was immunonegative for Bax throughout the entire prenatal period, while Bak immunoreactivity was seen only in the olfactory bulb neuroepithelium towards the end of fetal life. These findings are consistent with the delayed and prolonged neurogenesis which has been reported for the olfactory bulb,⁴⁰ and with the prolonged survival of the subventricular zone in the olfactory recess of the lateral ventricle. The initiation of apoptosis may be a delayed event in the olfactory bulb, as indicated by the absence of Bax immunoreactivity. The Bak positive cells in olfactory bulb and the striatum area are of interest because they lie along the route of the olfactory migratory stream, which supplies the olfactory bulb with neurons during postnatal life. The high level of Bid, particularly in the differentiated mitral cells, was insufficient for apoptosis in the olfactory region, which is in agreement with the recent evidence from *bid* knockout mice³⁵ and with knowledge that the Bid protein is normally inactive until cleaved by caspases.²³

The pattern of distribution of Bcl-2, Bax, Bcl-X_L and Bid in the prenatal hippocampus is in essence similar to the distribution in the developing cerebral cortex. In both structures during midfetal life, Bcl-2 is distributed throughout most of the thickness of the cortical plate, Bcl-X_L and Bid are preferentially distributed in the superficial layers of the cortical and hippocampal plates, and Bax is observed mainly in the intermediate zone of both regions.

Among the differentiating neurons of the striatum, globus pallidus, ventral striatum and olfactory tubercle, where Bcl-2 and Bax levels are low but Bcl-X_L and Bid levels are high, the balance of Bcl-2 family gene signaling may assure a period of differentiation undisturbed by initiation of apoptosis.

The possibility of a role for Bcl-2 family proteins in aspects of development other than apoptosis may be relevant to the data reported here. There is only a poor correlation between Bax, Bak, Bid and TUNEL labeling throughout development of the brain, probably because other factors beside the expression of these proteins may play a role in determining the fate of particular cells. Strong prenatal labeling with the Bcl-2 family members, particularly Bcl-X_L is more consistent with an involvement of these markers in neurogenesis and the early stages of differentiation, rather than apoptosis, as assessed in these areas by TUNEL assay. Moreover, changes in Bcl-2 family gene expression were temporally correlated with well-known events in differentiation, suggesting that Bcl-2 protein family

expression could be triggered by direct cell–cell interaction (migration) or interactions at terminal synaptic regions.

Two groups have independently provided evidence that Bcl-2 can prevent axon degeneration following fiber transection in the optic nerve track and in axotomized dorsal root ganglia.^{41,42} This influence on axon integrity appears to be separable from its effects as a suppressor of cell death, implying a novel function of Bcl-2 and, by analogy, Bcl-X_L. Based on these observations, therefore, it is tempting to speculate that Bcl-2 and Bcl-X_L expression in post-mitotic neurons after completion of migration reflects the participation of these proteins in the maintenance of axons within cells that have successfully established synaptic connections.

In addition to its effects on cell life span and axon integrity, Bcl-2 and Bcl-X_L have also been reported to inhibit cell proliferation, primarily by delaying the entry of quiescent G₀-phase cells into G₁-phase of the cell cycle.³ Though the mechanism by which Bcl-2 and Bcl-X_L suppress proliferation remains to be elucidated, mutagenesis experiments have shown that this anti-proliferative function is separable from the anti-apoptotic activity.⁴³ The finding that Bcl-2 and Bcl-X_L expression increase in post-mitotic neurons therefore raises suspicions about the potential participation of these Bcl-2 family proteins in the process of exit from the cell cycle and the preservation of the long-lived post-mitotic phenotype of differentiated neurons. Of these two anti-apoptotic proteins, gene knock-out experiments revealed that Bcl-X_L is of greater importance for maintaining neuronal cell homeostasis, since Bcl-X_L but not Bcl-2 knock-out was lethal and associated with massive neuronal cell death.³³ Interestingly, Bax deficiency prevents cell death of immature neurons in the Bcl-X knock-out model, which is consistent with the idea of critical ratios of proapoptotic and anti-apoptotic Bcl-2 family proteins.⁴⁴

The data presented here provide a framework for future investigations of the biological roles of Bcl-2 family proteins in the developing and adult mammalian brain.

Materials and Methods

Animals

The timing and the distribution of the expression of several members of the Bcl-2 family were assessed in paraffin sections derived from embryos and postnatal mice of the NMRI, FVB or C57Bl/6 strains. All procedures were approved by the relevant institutional animal care committee. Prenatal development was studied on a closely spaced series of mouse embryos (at daily intervals from 4 days of gestation (E4) to E8 and half-day intervals from E8 until postnatal day 4 (P4)). Fifty-eight mice were studied at regular intervals after that time until adulthood. Mice were mated overnight, and the morning the vaginal plug appeared was designated as embryonal day 0.5 (E0.5). The day of birth was termed as postnatal day zero (P0). All embryos were taken from mice which had been killed by either over-dose of ether or carbon dioxide. At E4–9, the uterus was excised and fixed with the embryos *in situ*. For the later embryos, each embryo was dissected from the uterus, freed from the extra-embryonic membranes and immediately placed in the fixatives, either phosphate-buffered (pH 7.4, 0.1 M) 4%

paraformaldehyde, Bouin's fixative, or zinc-buffered formalin (Z-Fix; Anatech LTD, Battle Creek, MI, USA). Immersion time varied from 2 days for early stages to 5–7 days for fetal and postnatal specimens. Staging of embryos was performed on the basis of external measurements (crown–rump length), external morphology and the advent of organ development (TS=Theiler Stages;¹).

Antibodies, immunohistochemistry and DNA fragmentation assay (TUNEL)

Dewaxed sections of whole mouse embryos were exposed to polyclonal antibodies (PAB) generated against synthetic peptides and confirmed to be specific for Bcl-2, Bcl-X, Bax, or Bak. The preparation and characterization of antibodies specific for Bcl-2 (PABs #1632 and #1634), Bcl-X (PABs #1688, recognizing Bcl-X-short and #1695 specific for the long form), Bax, and Bak have been described previously.^{15,45–47}

Polyclonal anti-Bid antisera were generated in rabbits using recombinant protein immunogens. Wild-type mouse BID and BIDΔ1-55 were produced as GST fusion proteins from pGEX vectors using *Escherichia coli* BL21 (DE3) as the host strain, and affinity-purified.²⁶ New Zealand white female rabbits were injected subcutaneously with recombinant protein (0.1–0.15 mg protein per immunization) and 0.5 ml Freund's complete adjuvant (dose divided over 10 injections sites) and then boosted three times at weekly intervals followed by another 3–20 boostings at monthly intervals with 0.15 mg recombinant protein immunogen in Freund's incomplete adjuvant, before collecting blood and obtaining immune serum.

Tissue sections were immunostained using a diaminobenzidine (DAB)-based detection method as described in detail, employing either an avidin-biotin complex reagent (Vector Laboratories) or the Envision-Plus-Horse Radish Peroxidase (HRP) system (DAKO) using an automated immunostainer (Dako Universal Staining System).^{35,45} The dilutions of antisera typically employed were 1:1500 (v/v) for anti-Bcl-2, 1:2000 for anti-Bax, 1:1500 for anti-Bcl-X (1695 and 1688), and 1:4000 for anti-Bid. Sections were coverslipped with or without methyl green/hematoxylin counterstaining, and photographed with either Nomarski optics or conventional illumination. Positive internal controls were available in the form of lymphoid cells in the thymus and liver sinusoids. For all tissues examined, the immunostaining procedure was performed in parallel using preimmune serum to verify specificity of the results. Initial confirmations of antibody specificity also included experiments in which antiserum was preabsorbed with 5–10 μg/ml of either synthetic peptide immunogen or recombinant protein immunogen. The immunostaining results were arbitrarily scored according to intensity as 0, negative; 1⁺, weak; 2⁺, moderate; and 3⁺, strong.

On selected representative sections the detection of nuclei with fragmented DNA (terminal deoxynucleotidyl transferase (TdT) end-labeling ('TUNEL assay'; ApopTagTM, Oncor Inc.)) was performed as a single- or two-color analysis as described previously,⁴⁶ using the colorimetric substrates DAB and either VIP or SG (Vector Labs. Inc.) for TUNEL and Bcl-2 family proteins, respectively.

Immunoblot analysis of Bcl-2 family proteins in developing CNS

To further confirm the changes in Bcl-2 family protein expression observed by immunohistochemical methods and to verify the specificity of our antibodies for analysis of these tissues, immunoblot analysis was performed using total protein lysates from the cerebrum, cerebellum, and the spinal cord of mice during development and adulthood. The anti-Bcl-2, Bax, Bcl-X_L, Bak and Bid antibodies reacted

with the expected size bands in these assays, demonstrating the monospecificity of these antibody reagents. The specificity of Bcl-X_S selective #1688 polyclonal antiserum was demonstrated using cell lysates derived from a cell line that expresses both Bcl-X_L and Bcl-X_S.

Acknowledgements

We would like to thank Xiaokun Xiao, S Lensing-Höhn and M Kazimirek for technical assistance and R Cornell for manuscript preparation. This research was supported by a generous grant from the NIH (NS 36821) to S Krajewski. KWS Ashwell was a fellow sponsored by the Alexander von Humboldt Foundation (Germany). JM Zapata is currently supported by the Lady Tata Memorial Foundation.

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