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Diane E. Merry
Washington University

Deborah J. Veis
Washington University

William F. Hickey
Dartmouth College

Stanley J. Korsmeyer
University of Washington

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bcl-2 protein expression is widespread in the developing nervous system and retained in the adult PNS

Diane E. Merry[1], Deborah J. Veis[1], William F. Hickey[2] and Stanley J. Korsmeyer[1]

[1]Department of Medicine and Pathology, Howard Hughes Medical Institute, Washington University School of Medicine, St Louis, MO 63110, USA
[2]Department of Pathology, Dartmouth Medical School, Lebanon, New Hampshire 03756, USA

SUMMARY

Cell death is a common feature of neural development in all vertebrates. The bcl-2 proto-oncogene has been shown to protect a variety of cell types from programmed cell death. We have examined the distribution of bcl-2 protein in the developing and adult nervous systems. bcl-2 protein is widespread during embryonic development. Proliferating neuroepithelial cells of ventricular zones as well as the postmitotic cells of the cortical plate, cerebellum, hippocampus and spinal cord express bcl-2. Postnatally, bcl-2 is principally retained in the granule cells of the cerebellum and dentate gyrus of the hippocampus. bcl-2 expression in the CNS declines with aging. In the peripheral nervous system, neurons and supporting cells of sympathetic and sensory ganglia retain substantial bcl-2 protein throughout life. The widespread expression of bcl-2 in CNS and PNS neurons during embryonic development and its selective retention in the adult PNS is consistent with a role for bcl-2 in regulating neuronal survival. In addition, the expression of bcl-2 in some neuronal populations beyond the recognized period of cell death is suggestive of a role for bcl-2 beyond simply protecting neurons from developmental cell death.

Key words: bcl-2 protein, expression, nervous system, cell death, human, monkey

INTRODUCTION

bcl-2 is unique amongst proto-oncogenes in that it functions as a repressor of programmed cell death (for review, see Korsmeyer, 1992). The *bcl-2* gene was isolated from the breakpoint of the t(14;18) chromosomal translocation found in follicular lymphoma (Tsujimoto et al., 1985; Bakhshi et al., 1985; Cleary and Sklar, 1985). The juxtaposition of *bcl-2* with the immunoglobulin (Ig) heavy chain locus results in a marked deregulation and overexpression of bcl-2 RNA and protein (Graninger et al., 1987; Seto et al., 1988). Transgenic mice that bear a bcl-2-Ig minigene that recapitulates this translocation demonstrated extended B cell survival and an expansion of resting B cells (McDonnell et al., 1989). Other transgenic mice, which redirected bcl-2 to immature thymocytes, expanded the role of bcl-2 as an antidote to apoptosis (Sentman et al., 1991; Strasser et al., 1991). bcl-2 conferred resistance to glucocorticoids, radiation and T cell receptor-induced cell death, while it did not prevent negative selection events. Overexpression of bcl-2 in certain hematopoietic cell lines has been shown to inhibit programmed cell death following cytokine deprivation (Vaux et al., 1988; Hockenbery et al., 1990; Nunez et al., 1990). Recently, bcl-2 has also been shown to protect primary neuronal cell cultures (Allsopp et al., 1993; Garcia et al., 1992) as well as PC12 cells (Batistatou et al., 1993; Mah et al., 1993) from neurotrophic factor withdrawal-induced cell death. The mechanism by which bcl-2 delays or prevents the onset of programmed cell death is unknown. bcl-2 protein is an integral membrane protein that has been localized to mitochondria (Hockenbery et al., 1990) as well as other membranes (Chen-Levy et al., 1989; Alnemri et al., 1992; Jacobson et al., 1993).

A role for bcl-2 in regulating the naturally occurring cell death that takes place in tissues of virtually all organisms is suggested by the topographic distribution of bcl-2 protein (Hockenbery et al., 1991). Adult tissues that express bcl-2 are characterized by (1) renewing stem cell populations, such as those found in hematopoietic lineages and complex differentiating epithelium, (2) glandular epithelium and (3) long-lived postmitotic cell populations.

A hallmark of nervous system development is the extensive histogenetic cell death that takes place relatively late in maturation and results in the loss of 20-80% of all neurons. According to the neurotrophic theory of neuronal cell death (reviewed in Oppenheim, 1991), differentiated, postmitotic neurons that fail to compete successfully for target-derived neurotrophic factors die. Other factors that may influence this developmental cell death include electrical activity (Lipton, 1986), afferent stimulation (reviewed by Oppenheim, 1991) and local factors such as cell-cell and cell-matrix interactions (Walicke, 1989).

Given the evidence that bcl-2 is involved in controlling programmed cell death in several in vitro and in vivo systems, we have examined the involvement of bcl-2 in nervous system development. First, we investigated the expression of bcl-2

protein in the developing mouse brain. Next, we examined bcl-2 expression in the adult brain in order to identify the cell types retaining bcl-2 expression. Finally, we analyzed bcl-2 expression in the peripheral nervous system, which unlike the central nervous system, displays the capacity in the adult to regenerate following injury.

MATERIALS AND METHODS

Two Rhesus monkey brains (16 and 18 years old) were obtained at autopsy (from naturally expired animals kept at the Bethesda Eye Institute, St Louis, MO). Human frontal cortex was obtained from surgical biopsy material from a 6-year-old girl. Tissue was snap-frozen in OCT without prior fixation. Frozen sections (5 μm) were fixed in methanol at −20°C for 2 minutes, rinsed in PBS (for mouse staining) or 0.5 M Tris-HCl (for monkey and human staining), blocked with avidin and biotin (Vector Laboratories) for 10 minutes each, blocked with PBS containing 1% bovine serum albumin, 0.2% dry milk, and 0.3% Triton X-100 (for mouse staining) or 0.5 M Tris-HCl containing 1% fetal bovine serum (for monkey and human staining) for 15 minutes, and incubated with the primary antibody overnight at 4°C. A hamster anti-human bcl-2 monoclonal antibody, 6C8 (Hockenbery et al., 1990), was used on monkey and human sections at a concentration of 50 μg/ml; mouse sections were stained with one of three anti-bcl-2 antibodies: (1) monoclonal hamster anti-mouse bcl-2 antibody 3F11 (used at 15 μg/ml), (2) monoclonal rat anti-mouse bcl-2 antibody 4C11 (used at 100 μg/ml)(a gift from Dr Gabriel Nunez, Ann Arbor), or (3) polyclonal rabbit anti-mouse bcl-2 antibody B1 (used at 80 μg/ml)(a gift from Dr Gabriel Nunez, Protein A purified). Monoclonal antibody 3F11 was produced in an Armenian hamster using recombinant full-length mouse bcl-2 (Veis et al., 1993). Monoclonal rat and polyclonal rabbit anti-mouse bcl-2 antibodies were made by injecting animals with recombinant mouse bcl-2 fusion protein (mouse bcl-2 containing a C-terminal 22 amino acid deletion fused to glutathione-S-transferase; GST gene fusion system, Pharmacia). Negative control antibody was a monoclonal hamster anti-human tumor necrosis factor antibody (TN3)(a gift from Dr Robert Schreiber, St Louis, MO) for the hamster monoclonals, preimmune serum (also Protein A purified) for the rabbit antibody and blocking buffer alone for the rat monoclonal antibody. Following primary antibody incubation, slides were incubated with either a biotinylated goat anti-hamster IgG, biotinylated goat anti-rat IgG, or biotinylated goat anti-rabbit IgG (CalTag, Inc.) for 30 minutes at room temperature, followed by an incubation with biotinylated swine anti-goat IgG (CalTag, Inc.) All secondary and tertiary antibodies were absorbed against murine antigens by the manufacturer or by us, using mouse liver acetone powder. Sections were quenched for endogenous peroxidase activity by treating with 0.18% hydrogen peroxide in methanol for 15 minutes at room temperature, then incubated with horseradish peroxidase-conjugated streptavidin (Zymed, Inc.) for 30 minutes before developing with diaminobenzidine (BioRad, Inc.). Sections were counter-stained with alcian blue/methyl green. Analysis was performed on a Zeiss or Nikon photomicroscope. For double-staining experiments, monoclonal mouse anti-neurofilament and anti-glial fibrillary acidic protein antibodies were generated by one of us (W. H.), and anti-CD45 monoclonal antibody T29/33 (Omary et al., 1980) was obtained from Dr Matthew Thomas (Washington University, St Louis). The monoclonal antibody 2H3 (against the 155×10^3 M_r neurofilament protein) was obtained from the Developmental Studies Hybridoma Bank, National Institute of Child Health and Development, and has been described (Dodd et al., 1988; Chisaka et al., 1992). For these experiments, primary antibodies and secondary detection systems were incubated with sections either simultaneously or sequentially. Secondary antibodies were absorbed with liver acetone powder to prevent cross-reactivity with the inappropriate primary antibodies and with each other. Cross-reactivity experiments were performed to ensure the absence of cross-reactivity. In addition, primary antibodies were incubated with sections alone, as well as in combination. FITC-conjugated streptavidin (Sigma) was used following the tertiary antibody incubation, and a Texas Red-conjugated horse anti-mouse IgG was used for cell-type-specific antibodies. Western blotting was performed as previously described (McDonnell et al., 1990). After incubation with streptavidin-peroxidase conjugate (Zymed), development was with diaminobenzidine, enhanced with 0.03% nickel chloride (B1), or with enhanced chemiluminescence (ECL, Amersham) (3F11, 4C11).

RESULTS

bcl-2 expression in the central nervous system

Developmental expression

During embryonic and early postnatal development, bcl-2 protein can be detected in two different populations of cells: neuroepithelial cells of ventricular zones and postmitotic cells of several defined regions, including the cortical plate, developing cerebellum, hippocampus and spinal cord.

Ventricular zone and cortical plate

At E10.5 in the mouse, the nervous system consists entirely of proliferating neuroepithelial cells. bcl-2 expression appears uniform in these cells throughout the CNS, except in the mitotic zone (Bayer and Altman, 1991) immediately adjacent to the ventricles where a large number of unstained cells can be seen (Fig. 1A; also E12.5, Fig. 1B). A proliferating population of neuroblasts (the ventricular zone) surrounding the ventricles is maintained as the brain develops. bcl-2 is expressed in the ventricular zone until this layer disappears around the time of birth (Fig. 1B-D); however, expression in the subventricular zone, an additional proliferative zone, can still be seen at postnatal day 11 (P11) (not shown).

At about E14 in the mouse, cells begin to differentiate and migrate out of the ventricular zone to form the cortical plate. The cortical plate is a densely packed zone of postmitotic cells, which are beginning to send out processes and form synaptic connections; it is most prominent around E16.5. bcl-2 expression appears uniformly high in this region (Fig. 1C). In whole embryo sections, the levels of bcl-2 expression in this layer are consistently higher than any other site in the body (Veis et al., unpublished data). At high power, bcl-2 staining is very intense and covers the entire cell, including the nucleus. Cells in the process of migrating from the ventricular zone to the cortical plate comprise the intermediate zone; bcl-2 expression is low or absent in these migrating cells (Fig. 1C,D). From E14.5 to E16.5, cells in the ventricular zone contain more bcl-2 than those in the adjacent intermediate zone, but less than those in the cortical plate. By E18.5, many cells within the cortical plate have differentiated to form the cortical layers found in the adult. bcl-2 staining in the cortical plate is less intense and less homogeneous at this time (Fig. 1D). At P3, many cells in the cortical gray still express bcl-2, although the staining has decreased further on a cell-to-cell basis, as shown in Fig. 1E. Interestingly, subplate neurons, seen in Fig. 1C-E, express high levels of bcl-2 throughout this time. By P11, a further decrease in bcl-2 staining in the cortical gray is noted (data not shown).

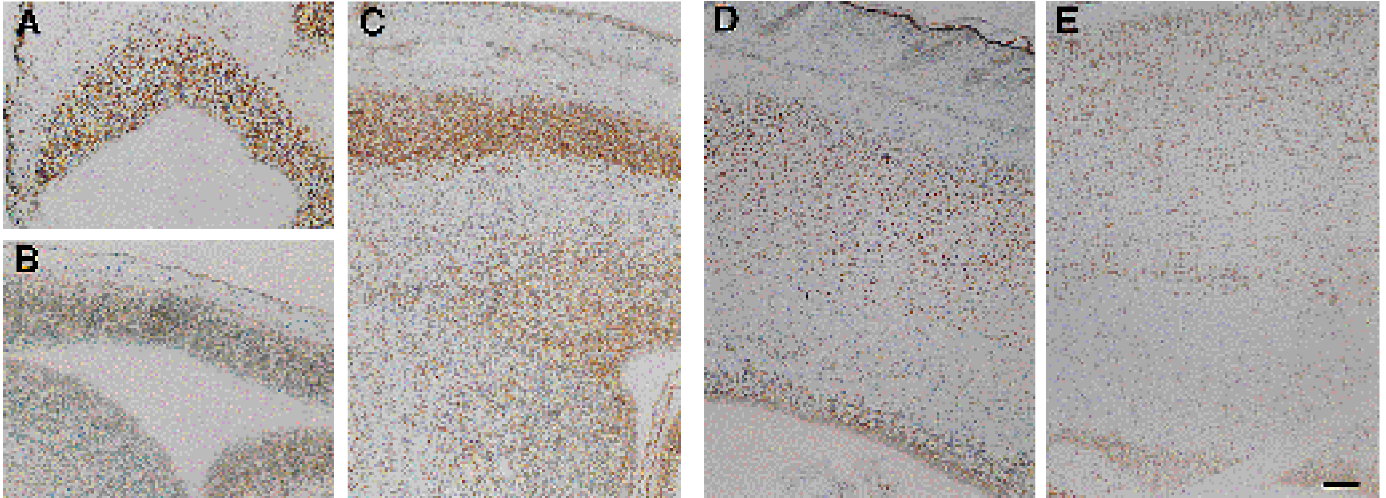

Fig. 1. bcl-2 expression in the developing cortex. (A) E10.5 mouse forebrain stained with 3F11. (B) E12.5 mouse brain surrounding lateral ventricle stained with 3F11. The surface of the head is at the top, with thalamus at the lower left. (C) E16.5 mouse brain oriented as in B, stained with 3F11. Apparent staining of the choroid plexus is an artifact. Note that cells within the thalamus do not stain for bcl-2 (as in B). (D) E18.5 brain superficial to the lateral ventricle. (E) Cortical gray at P3 stained with 3F11. Surface of the brain is at the top. Scale bar, 100 μm.

Cerebellar cortex

A dynamic pattern of bcl-2 expression is observed in the developing cerebellar cortex. During the development of the cerebellar cortex (E14-E15), neurons arise from two different ventricular zones, Purkinje cells from the ventricular germinal zone in the fourth ventricle and cerebellar local-circuit neurons (granule cells, stellate cells, and basket cells) from the external germinal layer (EGL), a layer of cells covering the surface of the cerebellum that originates from cells of the fourth ventricle. The EGL starts as a single cell layer and proliferates to form a layer six to eight cells deep. Two subpopulations of cells exist within the EGL (Ramon y Cajal, 1911), an external cycling population (EGL_a), and an internal differentiating layer (EGL_b) (Altman, 1972; Kuhar et al., 1993). Up to 3% of postmitotic neurons in the EGL die before P21 in the developing mouse cerebellum (Jacobson, 1991).

At E18.5 (Fig. 2A), bcl-2 is expressed in the cerebellar mantle layer, composed of developing Purkinje and glial cells, and in the deeper cells of the cerebellum. Strikingly, there is little or no bcl-2 expression in the developing external germinal layer. By P11, however, significant bcl-2 expression can be detected in the external germinal layer (Fig. 2B). Upon closer inspection, bcl-2 appears to be somewhat increased within the inner differentiating zone of the external germinal layer, EGL_b (seen best with antibody B1, Fig. 2F, but also seen reproducibly with 3F11). Cells within the developing internal granule cell layer clearly contain immunoreactive bcl-2 protein. At this time, the single row of Purkinje cells varies in its positivity for bcl-2. Differences in Purkinje cell staining with 3F11 and B1 probably relate to differences in epitope presentation with the fixation/staining protocol used here. Considerable expression of bcl-2 is seen in the mouse cerebellum throughout the first postnatal month (data not shown), beyond the time when cell death occurs within the EGL. However, by 5 months of age, the amount of immunoreactive bcl-2 is substantially decreased (Fig. 2D).

Hippocampus

The developing hippocampus also expresses bcl-2 (Fig. 3). This expression is widespread throughout the dentate gyrus and subiculum from E18.5 through P11 (Fig. 3A-D), but decreases substantially by P28 (Fig. 3E). Neuron production in most hippocampal regions is completed before birth in the mouse; however, in the dentate gyrus, neurons continue to be formed until P20 (Angevine, 1965; Bayer et al., 1980). Cell death within the differentiated neurons of the dentate gyrus is thought to occur between P20 and P27 (Wimer et al., 1988). Both the monoclonal antibody 3F11 and the polyclonal antibody B1 stain the inner cells of the dentate gyrus more intensely than the more differentiated outer cells (Fig. 3A,B). These inner cells of the developing dentate gyrus comprise a stem cell population that arises from cells of the lateral ventricle that migrate along the hippocampal fimbria to the inner region of the dentate gyrus.

The expression of bcl-2 in the hippocampus has diminished considerably by 5 months of age in the mouse, with only occasional cells of the dentate gyrus staining with the antibody 3F11 (Fig. 3F). Given that neurogenesis may continue in the adult dentate gyrus (Kaplan and Hinds, 1977), these cells may represent a functionally distinct subset.

Confirmation of the immunohistochemical data in cerebellum and hippocampus was obtained by Western analysis of protein lysates from P10 cerebellum and hippocampus using 3F11 and 4C11 monoclonal and B1 polyclonal antibodies (Fig. 4).

Spinal cord

Considerable cell death (as much as 60-70%) takes place during the development of spinal motoneurons (Lance-Jones, 1982; Oppenheim, 1986; Oppenheim et al., 1986). In addition, cell counting studies in the mouse (Lance-Jones, 1982) indicate that cell deaths in the alpha motoneurons of the lateral motor column occur between E13 and E18. Essentially no cell death within this neuron population takes place after birth (Lance-

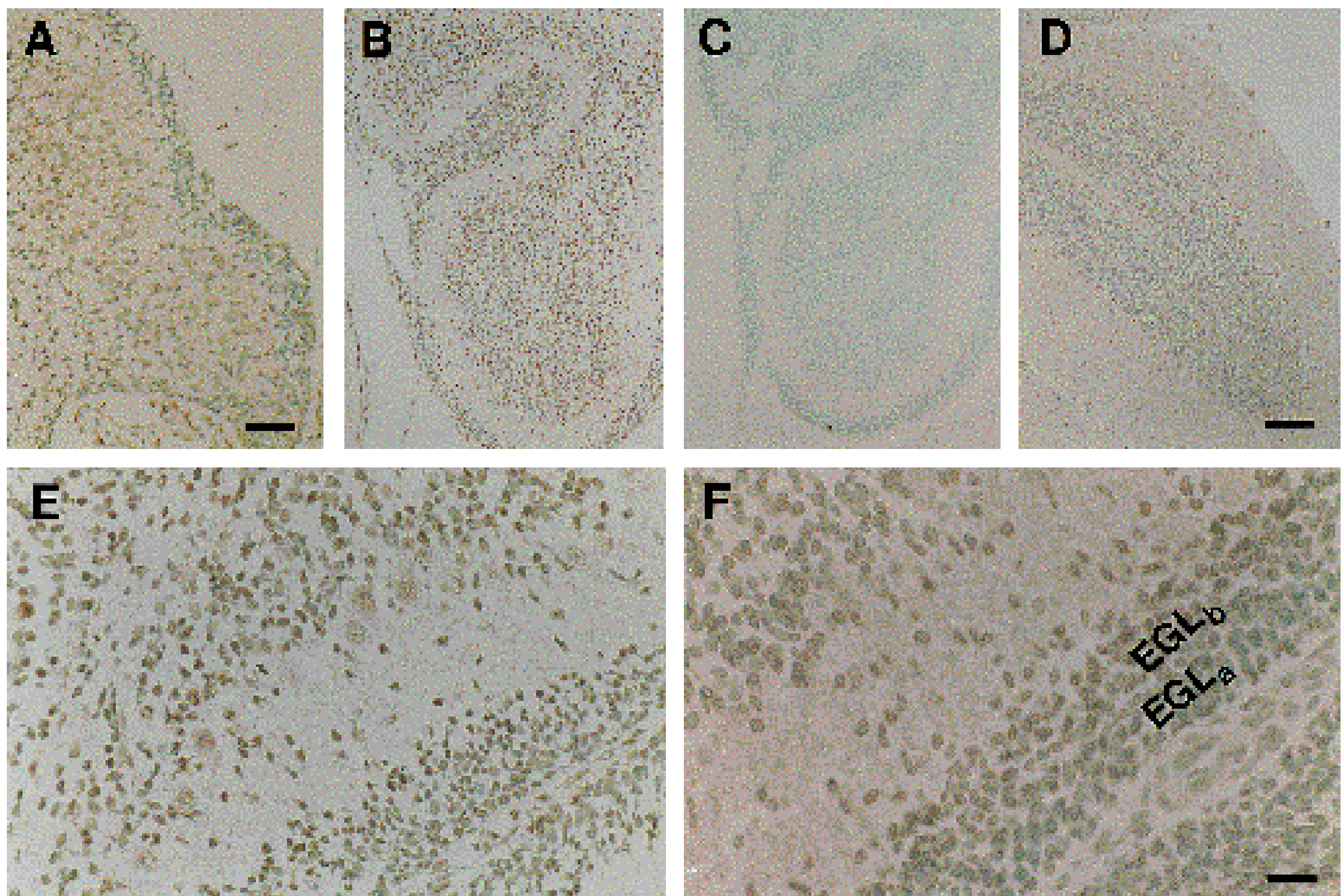

Fig. 2. bcl-2 expression in mouse cerebellum. (A) E18.5 cerebellum, stained with 3F11. The external germinal layer is relatively negative, while the mantle zone, composed of differentiating Purkinje cells, glial cells, and some granule cells is positive. Scale bar, 50 µm. (B) P11 cerebellar cortex, stained with 3F11. (C) Adjacent P11 section stained with control TNF antibody. (D) 5 month cerebellar cortex, stained with 3F11. Scale bar, 100 µm. (E) Higher magnification of B. (F) P11 cerebellar cortex, stained with the polyclonal antibody B1. Egl$_a$ designates the outer proliferating zone of the external germinal layer; Egl$_b$ designates the inner early differentiating cells of the external germinal layer. Note the increased bcl-2 staining in the Egl$_b$ compared to Egl$_a$. Scale bar, 25 µm.

Jones, 1982; Oppenheim, 1986). For these reasons, we investigated the pattern of bcl-2 expression in the developing mouse spinal cord from days E13.5 through E18.5. In the lumbar spinal cord at E13.5 (Fig. 5A,D), bcl-2 expression is greater within the differentiating neurons of the mantle layer than in the proliferating neuroblasts surrounding the neural canal. The motoneurons of the developing lateral motor column express even more bcl-2 than surrounding cells. At E16.5 (Fig. 5B,E), bcl-2 expression is seen in many cells throughout the mantle zone of the spinal cord; this staining is fairly heterogeneous. As seen at E13.5, bcl-2 expression is higher within the alpha motoneurons of the developing lateral motor column than in surrounding cells. The requirement for the use of frozen sections in these experiments prevents the identification of dying neurons. Therefore, we were unable to assess the expression of bcl-2 in those neurons undergoing programmed cell death. At embryonic day 18.5 (Fig. 5C,F), bcl-2 expression within the lumbar spinal cord is substantially the same as that seen at E16.5, with heterogeneous staining throughout the mantle layer. Again, somewhat higher bcl-2 expression can be seen in the motoneurons of the lateral motor column. These data show continued expression of bcl-2 in the alpha motoneurons of the lateral motor column seen in lumbar (Fig. 5) and cervical (not shown) spinal cord before, during and after the period during which the majority of these neurons die. While we cannot address the expression of bcl-2 in dying neurons of this population, we can say that it is retained in cells that survive. Postnatal expression of bcl-2 in the spinal cord is substantially decreased. By two months, there is considerably less bcl-2 protein overall; nonetheless, many cells in the dorsal, medial and ventral aspects of the cervical spinal cord still express bcl-2 (Fig. 4G-I). At 5 months, even fewer neurons express bcl-2 (not shown). Motor neurons of the adult spinal cord, in addition to neurons of the PNS (discussed below) are capable of regenerating their axons following injury. The continued expression of bcl-2 in some large ventral horn neurons may relate to this capacity.

Olfactory bulb and nasal epithelium

As with the cerebellum and hippocampus, the olfactory bulb represents a neuronal population that undergoes postnatal neurogenesis, particularly of granule cells (Jacobson, 1991). At P11 in the mouse, bcl-2 expression can be seen in mitral cells and granule cells (the first- and second-order neurons, respec-

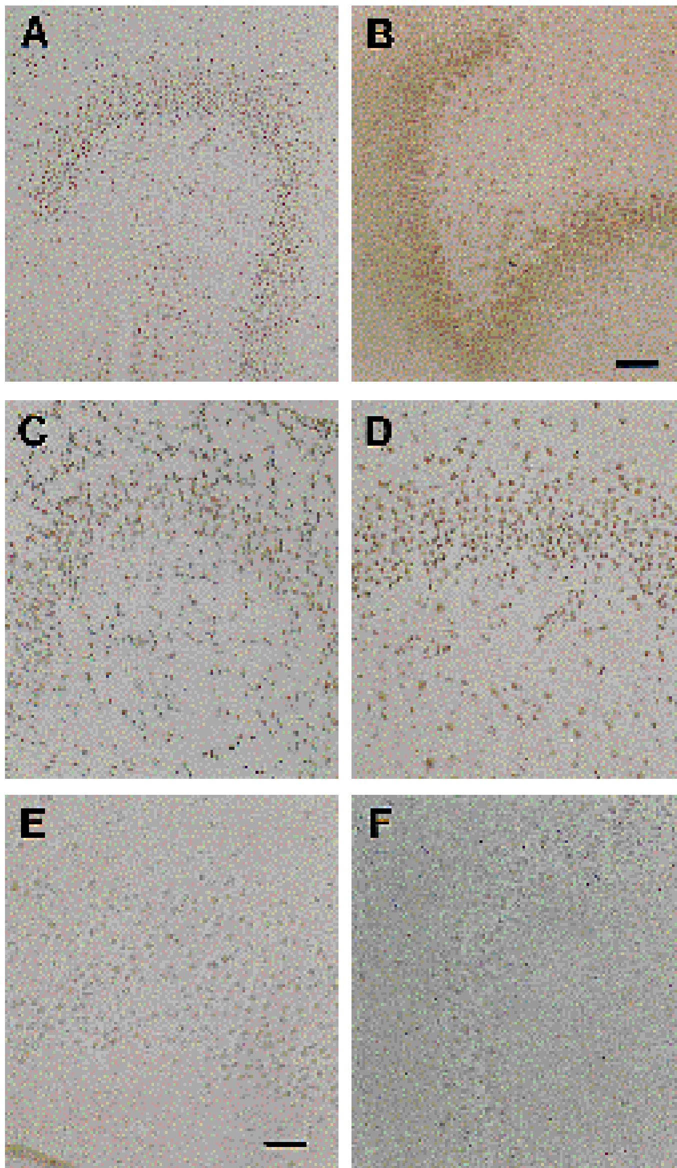

Fig. 3. bcl-2 expression in the hippocampus. Dentate gyrus of a P11 mouse stained with 3F11 (A), and the polyclonal antibody B1 (B). Note that the staining appears to be greater within the innermost cells of the dentate gyrus (B), which at this time represent a stem cell population. Higher magnification of the dentate gyrus is seen in (C) at E18.5, (D) at P11, (E) at P28, and (F) at 5 months. Staining in C-F is with 3F11. Scale bar in A, B and F, 100 μm. Scale bar in C, D and E, 50 μm.

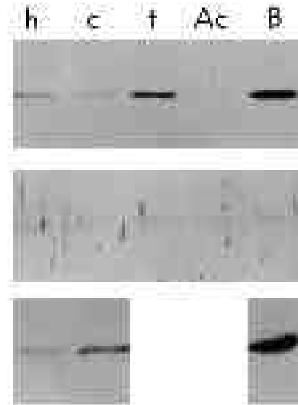

Fig. 4. Western blots of P10 hippocampus (h), cerebellum (c) and thymus (t), adult cerebellum (Ac) and a pro-B cell line FL5.12 (B) reacted with 3F11 (top), B1 (middle), and 4C11 (bottom). The $25{\times}10^3$ M_r bcl-2 protein is the only protein specifically detected by all antibodies.

tively, of the olfactory bulb), as well as within the glomeruli (which contain synapses from olfactory neurons on mitral cell dendrites) of the main olfactory bulb (Fig. 6A). In addition, bcl-2 protein is apparent within the cells of the accessory olfactory bulb (AOB) at this time (not shown). Neurogenesis is thought to continue in the olfactory bulb in the adult (Kaplan and Hinds, 1977; Bayer, 1983). At 5 months (Fig. 6B), substantial bcl-2 expression can be detected in the mitral cells (the first-order neurons of the olfactory bulb) as well as in the glomeruli (not shown). Granule cell expression appears to be decreased relative to P11. Expression within the accessory olfactory bulb also remains high (Fig. 6C), specifically within the glomeruli and the stellate cells (the first-order neurons of the AOB).

The olfactory epithelium represents a unique neuronal population that continues neurogenesis in the adult. It is similar to the epidermis in many aspects of its differentiation (Mahanthappa and Schwarting, 1993). Given the continued neurogenesis in this structure, along with the finding of bcl-2 expression in olfactory bulb glomeruli in the adult (Fig. 6C), we examined adult olfactory epithelium for bcl-2 expression. As shown in Fig. 6D, bcl-2 protein can be seen in differentiating neurons of the olfactory epithelium. Substantially less immunoreactive bcl-2 can be seen in stem cells (arrowhead) than in the differentiating neurons above. bcl-2 can also be seen in the Schwann cells surrounding the axon bundles located below the epithelial layer.

Adult expression patterns

By 5 months of age in the mouse, the amount of neuron-specific bcl-2 expression has substantially decreased. Cerebellar granule cells and granule cells of the dentate gyrus express little or no bcl-2 at 5 months (compare P11 to 5 months in Figs 2B versus D, 3C versus F), and spinal cord neurons express substantially less bcl-2 than at birth (compare E18 to 2 months, Fig. 5C,F versus G,I).

To further address bcl-2 expression in the adult, we analyzed 16- and 18-year-old Rhesus monkey brains using the anti-human bcl-2 antibody, 6C8. The following regions were analyzed: pituitary, medulla, pons, substantia nigra, cerebellar cortex, visual cortex, parietal cortex, frontal cortex, temporal cortex, anterior and posterior thalamus, hypothalamus, hippocampus, globus pallidus, caudate and putamen. A survey of the monkey brain in this fashion showed that the vast majority of bcl-2 protein in the adult brain resulted from microglial expression. In addition, immunostaining of human frontal cortex obtained at surgical biopsy revealed identical findings. Fig. 7 shows the results of immunohistochemistry experiments using 6C8 alone and in combination with two cell type-specific antibodies. The microglial pattern of expression is documented in Fig. 7A and C. Two-color immunofluorescence (Fig. 7C-F) revealed that virtually all of the bcl-2-positive cells in human frontal cortex also co-express the microglial marker CD45. Within the central nervous system, CD45 expression is seen in microglia, macrophages found in meninges and around blood vessels, and in rare lymphocytes (Lassman et al., 1991). In this

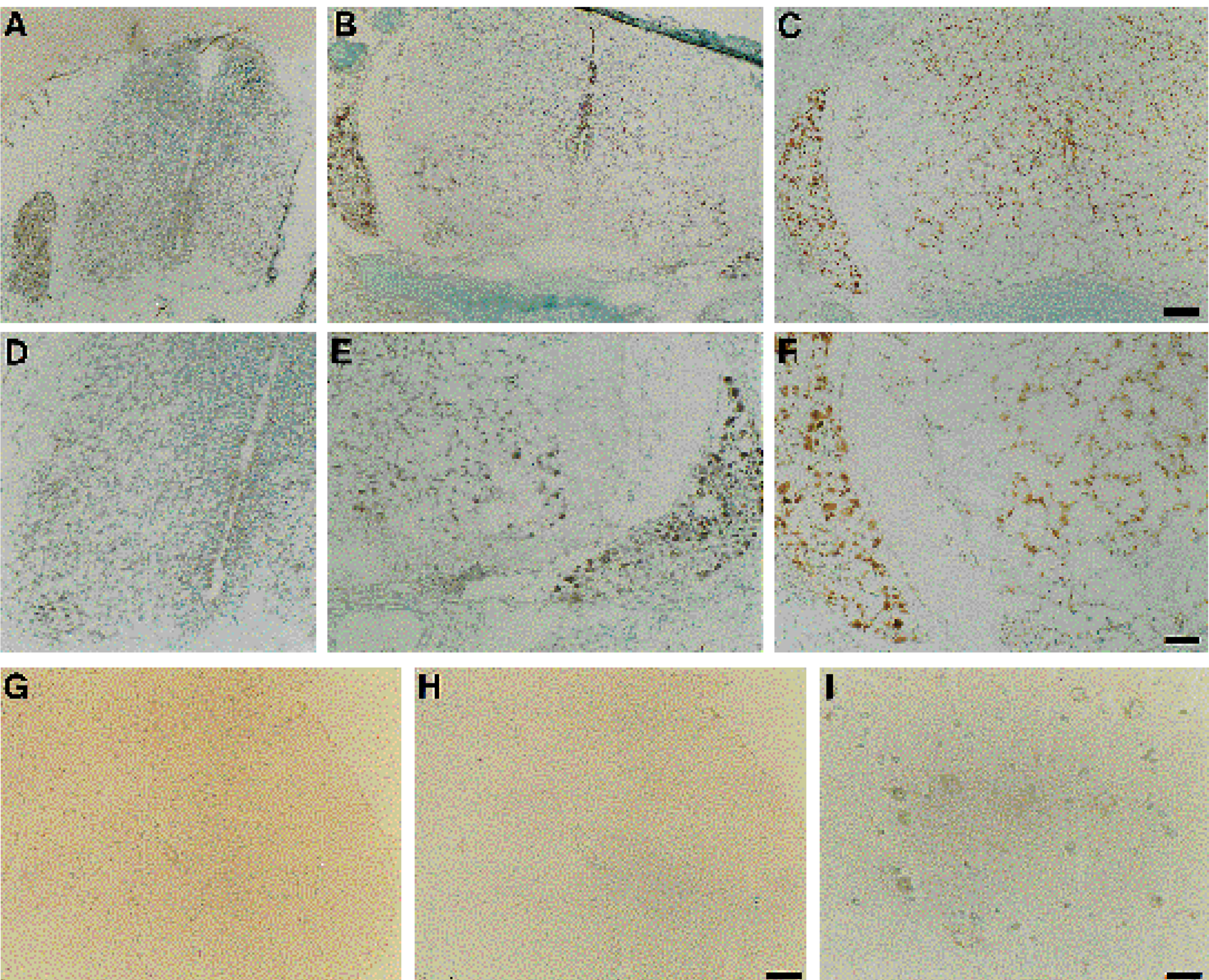

Fig. 5. Expression of bcl-2 in the developing spinal cord with 3F11. (A,D) Horizontal section of E13.5 lumbar spinal cord. bcl-2 staining appears highest within the differentiating neurons of the mantle zone, and is most intense in the motoneurons of the developing lateral motor column. (B,E) Horizontal section of E16.5 lumbar spinal cord. The staining appears more hetergeneous that at E13.5. Highest expression is seen in the neurons of the ventral horn. (C,F) Horizontal section of E18.5 lumbar spinal cord stained with 3F11, showing similar staining to that seen at E16.5. Note also the staining of DRG neurons during this time. Scale bar for A-C, 100 µm. Scale bar for D-F, 50 µm. (G-I) Horizontal sections of two month cervical spinal cord stained with 3F11 (G,I) or TNF (H). Scale bar for G,H, 100 µm; scale bar for I, 25 µm.

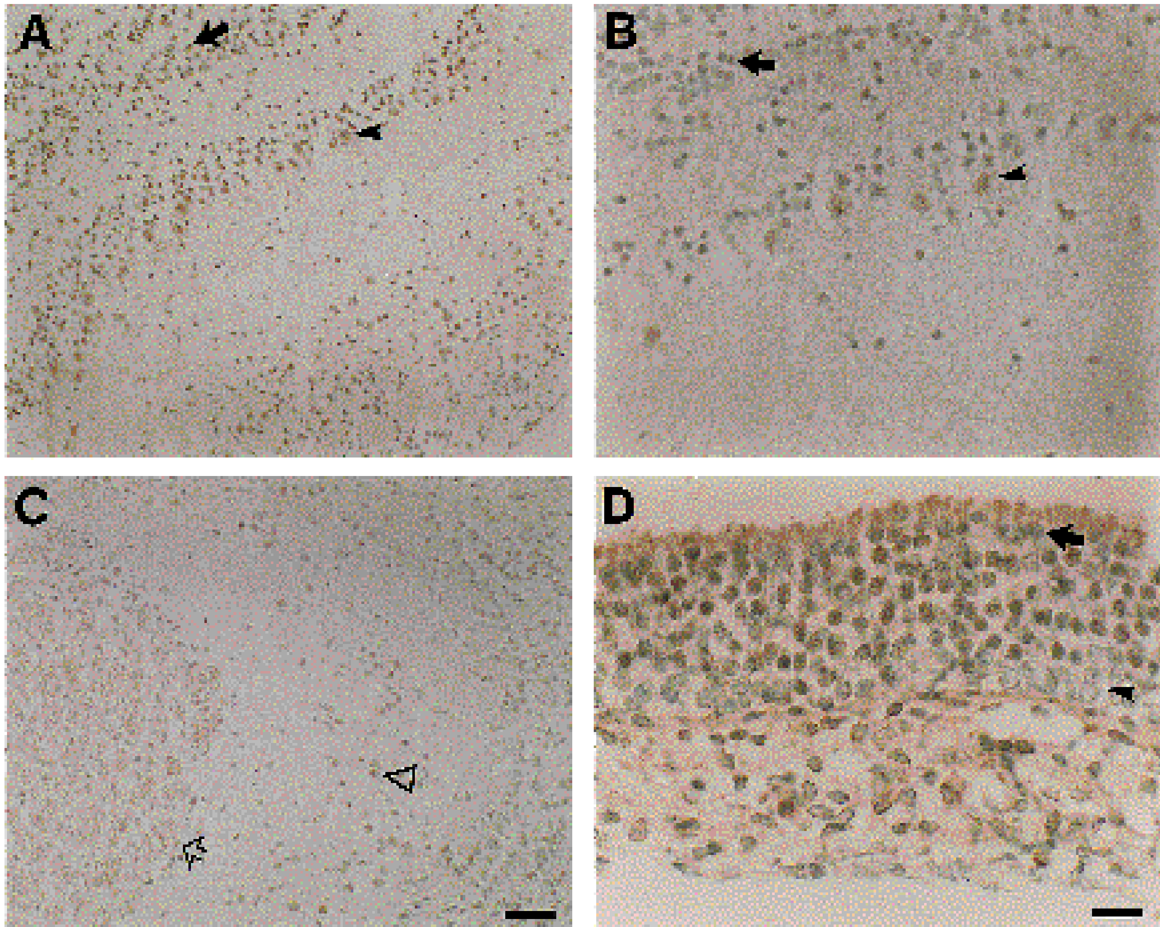

Fig. 6. bcl-2 staining in the olfactory bulb (A-C) and olfactory epithelium (D). (A) Horizontal section of P11 olfactory bulb stained with 3F11. Both mitral (arrowhead) and granule (arrow) neurons are positive at this time. (B) Horizontal section of 5 month olfactory bulb. The first-order mitral cells (arrowhead) are positive, while the granule cells (arrow) show reduced expression. (C) Accessory olfactory bulb. The stellate neurons (open arrowhead), the first-order neurons in this structure, are positive, as are glomeruli (open arrow), which contain synapses from olfactory neurons. Glomeruli of the main olfactory bulb are also positive (not shown). (D) Adult olfactory epithelium stained with 3F11. Note that the basal cells (arrowhead) are negative, while the differentiating neurons located above the basal cells are positive. The sustentacular cells (arrow), located next to the outermost ciliated bulbs (which contain the dendrites of the most mature neurons) appear less intense than the neurons. Scale bar in A and C, 50 μm. Scale bar in B and D: for B, 25 μm; for D, 12.5 μm.

context, microglial cells are substantially more abundant than other CD45-expressing cells. Similar two-color immunofluorescence experiments using anti-glial fibrillary acidic protein antibody and 6C8 showed no bcl-2-positive astrocytes (not shown). The only cells of the adult monkey brain consistently exhibiting a non-microglial pattern of expression were the neuroendocrine cells of the anterior pituitary (Fig. 7B). However, while the majority of neurons did not express bcl-2 (Fig. 7E,F), rare, individual neurons exhibited both the 200×10^3 M_r neurofilament protein and bcl-2 (Fig. 7G,H). The functional difference between these and other neurons is unknown.

bcl-2 expression in the peripheral nervous system

The peripheral nervous system differs from the central nervous system in several ways, including its neural crest origin and its regenerative capacity following injury (reviewed by Johnson et al., 1986). We investigated expression of bcl-2 in three structures of the peripheral nervous system: the superior cervical ganglion, dorsal root ganglion and the adrenal gland.

Superior cervical ganglion

Expression of bcl-2 in neurons of the superior cervical ganglion is shown in Fig. 8. At P20 (Fig. 8A), substantial expression of bcl-2 can be seen in these postmitotic sympathetic neurons. In contrast to the temporal pattern of bcl-2 seen in the central nervous system, these sympathetic neurons remain strongly positive at 2.5 months (Fig. 8B) and 5 months (data not shown). Of note, a varied intensity of bcl-2 is observed in different sympathetic neurons.

Dorsal root ganglion

Fig. 9 shows the expression of bcl-2 in the mouse dorsal root ganglion (DRG) at E18.5, P20 and 5 months of age (Fig. 9A,C and E, respectively). It is evident from this figure that bcl-2 expression in these neurons remains high throughout life. Consistent with this finding, expression of bcl-2 in the DRG of an 18-year-old Rhesus monkey can also be seen (Fig. 9G). In addition, during normal development, about half of the DRG neurons that are produced will die (reviewed by Jacobson,

1991). This process is known to be affected by the supply of target-derived neurotrophic factors, but may be influenced by other factors as well, since many neurons die before reaching their targets. bcl-2 is expressed in these neurons throughout the period of cell death (Fig. 5).

In addition to the typical cytoplasmic distribution of bcl-2 seen in most DRG neurons, a nuclear staining pattern is also seen with the 3F11 antibody. This nuclear staining pattern has been noted in developing neurons throughout the brain, as well as in some other organs using 3F11 (Veis et al., 1993). However, the staining pattern seen with 3F11 can appear strictly cytoplasmic within certain cell types (eg. medullary thymocytes). Immunostaining of mouse DRG with another monoclonal antibody, 4C11, is shown in Fig. 9F; here the staining is primarily cytoplasmic, consistent with the pattern seen with the anti-human bcl-2 antibody 6C8 (Fig. 9G). While we cannot exclude an artifact of histologic preparation, it is possible that bcl-2 also localizes to nuclei in certain cell types. 3F11 may recognize a different bcl-2 epitope than 4C11 or 6C8, that may be presented on nuclear as well as cytoplasmic bcl-2. It also remains possible that 3F11 recognizes an epitope on bcl-2 that is conserved in other bcl-2-related proteins (Bax or bcl-x) (Oltvai et al., 1993; Boise et al., 1993). However, it should be noted that the same cells that are identified as expressing bcl-2 with the monoclonal antibody 4C11 (Fig. 9F) and with the polyclonal antibody B1 (Fig. 2F) are also detected with 3F11; therefore any cross-reacting protein would have to be regulated in a pattern identical to bcl-2. In addition, in situ hybridization of adult DRG neurons with a full-length bcl-2 probe confirms the results shown here. Lastly, LeBrun et al. (1993) have shown bcl-2 protein in neurons of spinal ganglia, as well as in spinal cord and neocortex, confirming the results shown here. It is also important to note the expression of bcl-2 in the 'supporting cells' of the SCG and DRG, seen in Fig. 8A,B, and Fig. 9G. These cells include members of the macrophage/monocyte family which represent peripheral nervous system microglial equivalents (Vass et al., 1993).

Two-color immunofluorescence studies of E18.5 dorsal root ganglia using 3F11 and the embryonic neurofilament antibody 2H3 reveal an interesting finding. The smallest neurons of the dorsal root ganglion, which express the most intense levels of bcl-2, are negative for 2H3 (Fig. 9B,D, arrow),

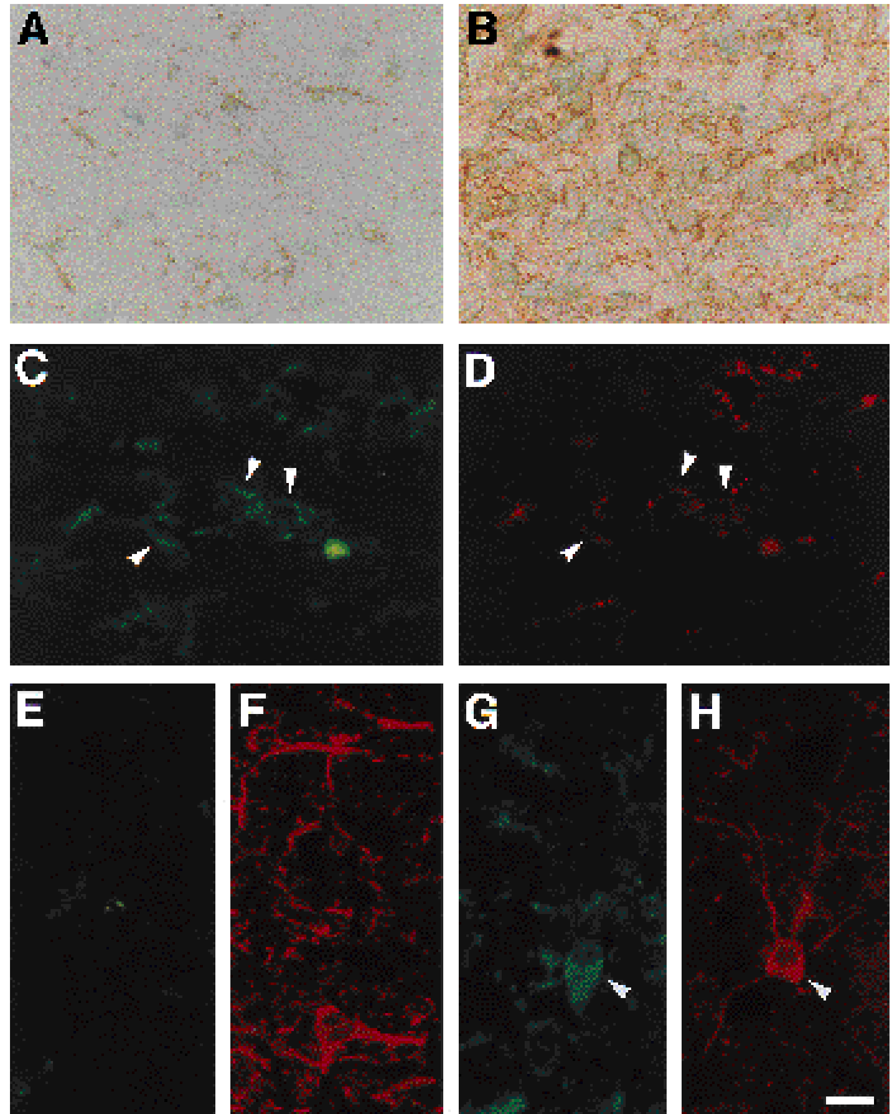

Fig. 7. bcl-2 expression in adult Rhesus monkey and human brain. (A) Staining of 18-year-old Rhesus monkey frontal cortex with 6C8. (B) bcl-2 expression in monkey anterior pituitary. (C,D) Two-color immunofluorescence of human frontal cortex with 6C8 (C) and the CD45 antibody T29/33, which identifies microglia (D). Note the coincident staining with the two antibodies. Representative cells are marked by arrows, but all anti-CD45-positive cells are positive for bcl-2. (E-H) Two-color immunofluorescence of human frontal cortex stained with 6C8 (E,G) and anti-NF$_H$ (F,H) antibodies. A rare neuron concordant for the two antibodies is seen in G and H. Scale bar, 25 μm.

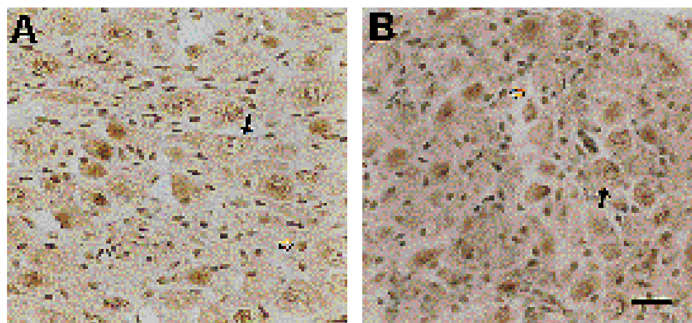

Fig. 8. bcl-2 expression in superior cervical ganglion (SCG). P20 (A) and 2.5 month (B) mouse SCG stained with 3F11. Neurons as well as supporting cells are positive for bcl-2. Filled arrows indicate representative neurons; open arrows indicate representative satellite cells. Scale bar, 25 μm.

whereas the larger neurons, which express bcl-2 at a lower level or in a more diffuse pattern, are positive for the neurofilament antibody (Fig. 9B,D, arrowheads). This is consistent with functional differences in these neurons (Carroll et al., 1992; Ruit et al., 1992; Johnson et al., 1980).

Adrenal gland

Expression of bcl-2 in the adrenal gland was observed in cells of both the cortex and medulla (not shown). Expression appears most intense in cortical cells, and within the cortex is highest in cells of the zona fasciculata. The expression of bcl-2 in the adrenal medulla and in other neurons of neural crest origin is consistent with the high level of bcl-2 expression seen in the vast majority of neuroblastomas (Reed et al., 1991; our unpublished observations).

DISCUSSION

The findings from the present study indicate that bcl-2 is more widely expressed in neurons of the developing brain than in those of the adult. High postnatal neuronal expression of bcl-2 in the brain is confined to those regions characterized by postnatal neurogenesis and differentiation (hippocampus, cerebellum, olfactory bulb), as well as to the specialized neuroendocrine cells of the anterior pituitary. However, bcl-2 expression in the peripheral nervous system remains high throughout life. The temporal and spatial patterns of bcl-2 expression in the developing central nervous system and in the developing and adult peripheral nervous system are consistent with a role for bcl-2 in protecting neurons from programmed cell death.

Two different populations of cells in the developing nervous system express bcl-2: proliferating stem cells of ventricular zones and postmitotic cells. bcl-2 is highly expressed in very early ventricular zones (E10.5, Fig. 1A) composed completely of dividing cells, and in postmitotic differentiating cells of many regions, including the cortical plate, cerebellar cortex and spinal cord. It may be that this diverse and widespread expression ensures the survival of an excess of neurons, on which subsequent cell death mechanisms can act to refine the number of differentiated, functional neurons to match synaptic targets.

The varied bcl-2 expression levels within dividing and postmitotic cells suggests a dynamic, regulated role for bcl-2; this

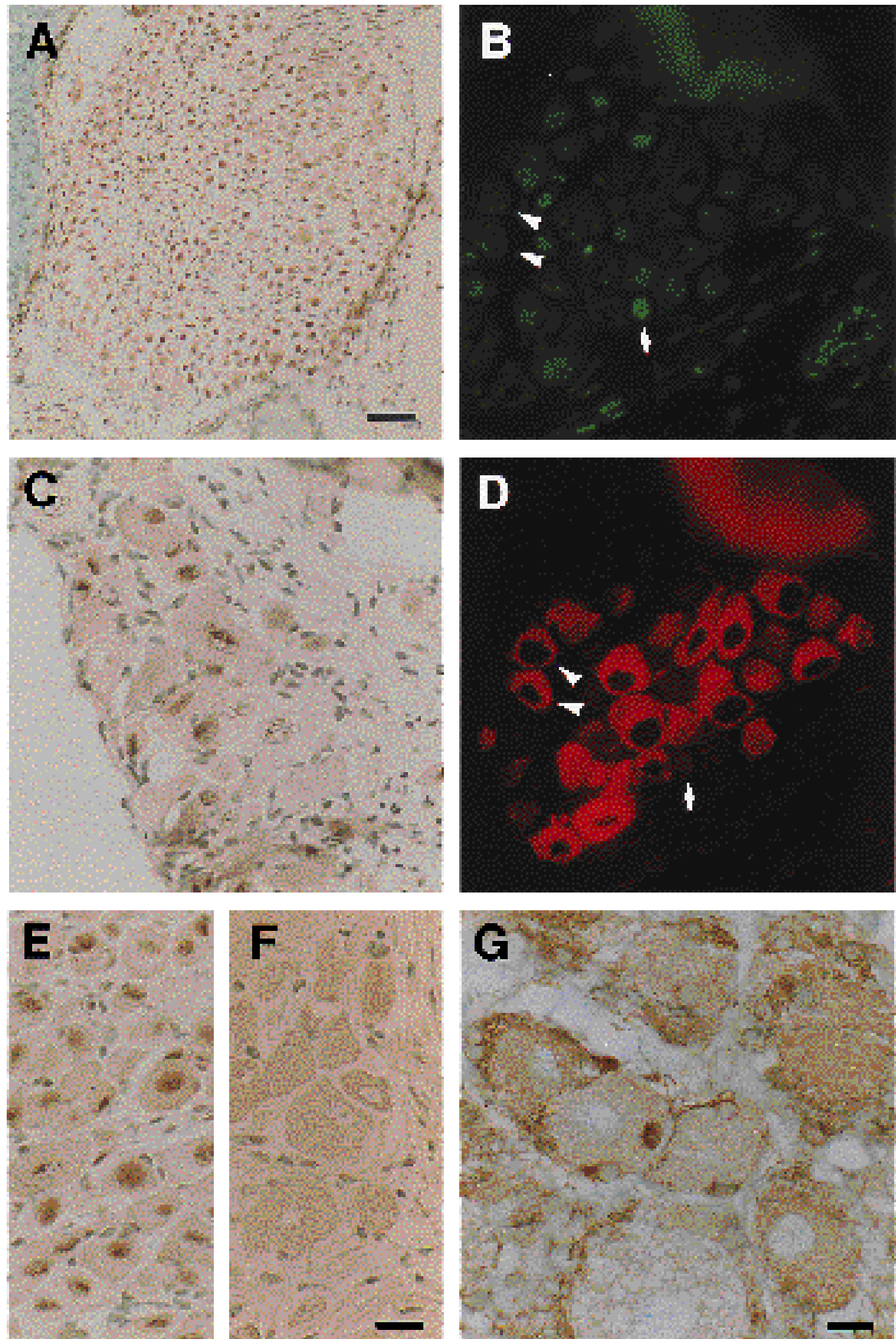

Fig. 9. Time course of bcl-2 expression in DRG. E18.5 (A), P20 (C), and 5 month (E,F) mouse DRG stained with 3F11 (A,C,E) and 4C11 (F). The small, dark neurons appear to express higher levels of bcl-2. Scale bar in A, 50 µm; scale bar for B-), 25 µm. (B,D) Two-color immunofluorescence of E18.5 DRG with 3F11 (B) and 2H3 (D). Note that the larger 2H3-positive neurons stain more weakly with 3F11 (arrowheads). This bcl-2 staining is above background (control antibody not shown). The small, dark bcl-2-positive neurons are negative for 2H3 (arrow). (G) 16 year Rhesus monkey DRG stained with 6C8. Note that 6C8 also stains satellite cells. Scale bar in G, 12.5 µm.

is best exemplified by the two cortical regions studied here. In the developing neocortex, the fate of neuronal precursors is determined while they are still proliferating within the ventricular zone (Bayer and Altman, 1991). It is intriguing that even in early ventricular zones (E10.5), there were bcl-2-negative cells in the mitotic zone that lines the ventricle (see Fig. 1A). The intermediate zone is markedly negative for bcl-2; however, whether this represents a change in bcl-2 expression within migrating cells or the more transient character of cells within this region is unclear. As differentiation proceeds in the neocortex, the differentiating cells of the cortical plate express higher levels of bcl-2 than the stem cells of the ventricular zone (Fig. 1C,D). The extent of neuronal death within the developing cerebral cortex remains unclear. The high expression of bcl-2 in this region, however, suggests a need for the protective effects of bcl-2. Alternatively, the expression of bcl-2 in the neocortex may indicate an additional role for bcl-2 in a commitment/differentiation process.

Within the developing cerebellum, as in the neocortex, bcl-2 protein is highest in postmitotic differentiating cells, particularly granule cells. While bcl-2 is present in the proliferating cells of the superficial EGL (termed EGL_a by Kuhar et al., 1993), the amount of bcl-2 protein increases in postmitotic granule cell precursors of EGL_b, which also express granule cell-specific differentiation markers such as TAG-1 (Dodd et al., 1988), astrotactin (Edmondson et al., 1988), and α-internexin (Kaplan et al., 1990). bcl-2 remains high in granule cells as they migrate through the developing molecular layer and begin to extend their fibers (the parallel fibers), and is retained in the fully differentiated cells of the internal granule cell layer. As in the cortical layers of the forebrain, a significant decrease in bcl-2 expression is noted in adult cerebellum, indicating a change in the regulation of bcl-2 with aging. This pattern of bcl-2 expression seen in developing cortical regions, as well as in the differentiated neurons of the adult olfactory epithelium, suggests that bcl-2 may play a role in the differentiation of some neurons of the developing nervous system. Alternatively, signals may exist in both dividing and differentiating cell populations to express bcl-2 and repress a cell death pathway.

bcl-2 expression in many brain regions is maintained at a high level throughout the period of cell death. As evidenced by the bcl-2 immunostaining in the spinal cord from E13.5 through E18.5 (a period during which cell death of alpha motoneurons takes place), there is no global decrease in bcl-2 expression in alpha motoneurons prior to the period of cell death. Surviving alpha motoneurons express somewhat higher levels of bcl-2 during this time than other spinal neurons; this may relate to their survival, or to additional characteristics of their differentiation. Other regions of the nervous system, including cerebellar cortex, dorsal root ganglia and the developing retina (Veis et al., unpublished data), reveal bcl-2 protein beyond the period when developmental cell death occurs. It may be that for a particular neuron, the failure to encounter its cognate neurotrophic factor(s) or receive adequate stimulation results in a decrease in bcl-2, allowing that neuron to progress along a death pathway. Alternatively, as mentioned, this extended expression of bcl-2 may indicate an expanded role for bcl-2 beyond protecting cells from death during development.

In contrast to the decline in bcl-2 expression seen in the brain with aging, the neurons of the peripheral nervous system (DRG, SCG and adrenal gland) continue to express high levels of bcl-2 protein in the adult. The neurons of the peripheral nervous system are capable of regeneration following injury and, as in developing neurons, the survival of peripheral neurons is dependent on target-derived neurotrophic factors. The high level of bcl-2 protein that is maintained in adult peripheral neurons is similar to its expression in developing neurons and suggests a specific role for this survival factor in both environments. In addition, the expression of bcl-2 in mature peripheral neurons suggests a differential regulation of bcl-2 protein compared with that found in neurons of the brain. This may provide a potential approach to identifying factors involved in the regulation of bcl-2 expression.

Within the aging brain, the decrease in bcl-2 protein is a gradual one, with some neurons of the hippocampus, cerebellum and cortex remaining positive well into postnatal life. This tapering of bcl-2 may be due to the decay of long-lived protein. Alternatively, it might also reflect a negative regulation of the bcl-2 promoter with maturation as has been demonstrated in B cell lines (Young and Korsmeyer, 1993). The decline of bcl-2 in CNS neurons might permit the neuronal loss seen in neurodegenerative diseases. Alternatively, the decline in bcl-2 in adult brain may be countered by expression of other bcl-2-like gene(s) or by genes involved in other cell death pathways (Allsopp et al., 1993). The established role of bcl-2 in protecting neurons from cell death (Garcia et al., 1992; Allsopp et al., 1993; Mah et al., 1993; Zhong et al., 1993; Batistatou et al., 1993), combined with the patterns of bcl-2 expression in neurons of the developing brain and adult peripheral nervous system, support efforts to define regulators of bcl-2 expression in an attempt to counter neurodegenerative cell loss.

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