Bcl-2 Accelerates the Maturation of Early Sensory Neurons

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Bcl-2 is a cytoplasmic protein that blocks apoptosis in a wide variety of cell types. Here we report a novel role for Bcl-2 in the early stages of neuronal development. Shortly after differentiating from progenitor cells, sensory neurons undergo a distinct morphological change; initially they have small, spindle-shaped, phase-dark cell bodies that become large, spherical, and phase-bright. Early sensory neurons cultured from the trigeminal ganglia of $bcl-2^{-/-}$ embryos at embryonic day 11 (E11) and E12 underwent this change more slowly than trigeminal neurons of wild-type embryos of the same ages. The delay was not attributable to the well documented role of Bcl-2 in preventing apoptosis, because Bcl-2-deficient early sensory neurons survived as well as wild-type neurons. Accordingly, there was a significantly smaller number of the more mature type of neuron in the early trigeminal ganglia of $bcl-2^{-/-}$ embryos, yet the number of neurons in the trigeminal ganglia of $bcl-2^{-/-}$ and wild-type embryos was similar. The absence of Bcl-2 did not cause a uniform delay in the developmental program of sensory neurons, because the time course of nerve growth factor receptor expression (both trkA and p75) was unaffected in the trigeminal neurons of $bcl-2^{-/-}$ embryos. These findings indicate that Bcl-2 expression is required for the normal progression of a particular early maturational change in embryonic sensory neurons.

Key words: Bcl-2; neuronal differentiation; apoptosis; neurotrophin; sensory neuron; gene-targeted mice

Bcl-2 is a widely expressed cytoplasmic protein that plays a key role in regulating cell survival in the immune system and nervous system. Mice with targeted null mutations in the *bcl-2* gene have markedly reduced numbers of B and T cells attributable to increased apoptosis (Nakayama et al., 1993, 1994; Veis et al., 1993), whereas mice carrying a transgene causing high levels of Bcl-2 expression in the immune system show extended survival of B and T cells (McDonnell et al., 1989, 1990). Overexpression of Bcl-2 in cultured neurons prevents their death after neurotrophin deprivation (Garcia et al., 1992; Allsopp et al., 1993b), and mice expressing a *bcl-2* transgene under the control of a neuron-specific enolase promoter have increased numbers of neurons in several regions (Martinou et al., 1994). The survival response of cultured cranial sensory neurons to neurotrophins during the phase of naturally occurring neuronal death is markedly reduced by antisense *bcl-2* RNA (Allsopp et al., 1995). Likewise, cranial sensory neurons from $bcl-2^{-/-}$ embryos do not survive as well *in vitro* with neurotrophins as wild-type embryos during the peak period of naturally occurring neuronal death and are lost to a greater extent than wild-type neurons during this period of development *in vivo* (Piñón et al., 1997). Sympathetic neurons from postnatal $bcl-2^{-/-}$ mice die more rapidly after nerve growth factor (NGF) deprivation *in vitro* than wild-type neurons (Greenlund et al., 1995), and postnatal $bcl-2^{-/-}$ mice have significantly fewer sensory, autonomic, and motor neurons than wild-type mice (Michaelidis et al., 1996). Furthermore, Bcl-2 is structurally and functionally similar to the *ced-9* gene product of *Caenorhabditis elegans* that also prevents programmed cell death (Hengartner and Horvitz, 1994).

In addition to the wealth of data indicating that Bcl-2 plays a key role in regulating cell survival, work on several cell lines has raised the possibility that Bcl-2 influences neuronal differentiation. Bcl-2 overexpression in a neural crest-derived line enhances neurite outgrowth and increases the expression of neuron-specific enolase (Zhang et al., 1996). Midbrain-derived dopaminergic lines stably expressing Bcl-2 extended longer neurites than control-transfected cells (Oh et al., 1996), and Bcl-2 enhances the differentiation of the PC12 cells grown in serum-free conditions (Batistatou et al., 1993).

Because the developmental significance of overexpressing Bcl-2 in cell lines is difficult to interpret, we compared the early development of newly differentiated sensory neurons of $bcl-2^{-/-}$ and wild-type mouse embryos. Shortly after sensory neurons differentiate from progenitor cells, they undergo a distinctive and clearly recognized morphological change; initially they have small, spindle-shaped, phase-dark cell bodies that subsequently enlarge and become spherical and phase-bright (Wright et al., 1992). We show that Bcl-2-deficient neurons retain a more immature morphology longer than wild-type neurons both *in vitro* and *in vivo*, and that this delay in early neuronal maturation is not a consequence of the antiapoptotic function of Bcl-2.

MATERIALS AND METHODS

Experimental animals. Bcl-2 null mutant mice were a gift of Dennis Loh, Nippon Roche Research Center, Kamakura, Japan (Nakayama et al., 1993, 1994). Embryos were obtained from overnight matings of $bcl-2^{+/-}$ mice. Pregnant females were killed at the required stage of gestation, and the genotypes of the embryos were determined by a PCR-based technique using DNA isolated from embryonic tissues. There were no consistent differences in the stage of development of wild-type and Bcl-2-deficient embryos in each litter using the staging criteria of Theiler (1972).

Neuronal cultures. Separate dissociated cultures of trigeminal ganglion neurons were established from each embryo in E11 and E12 litters
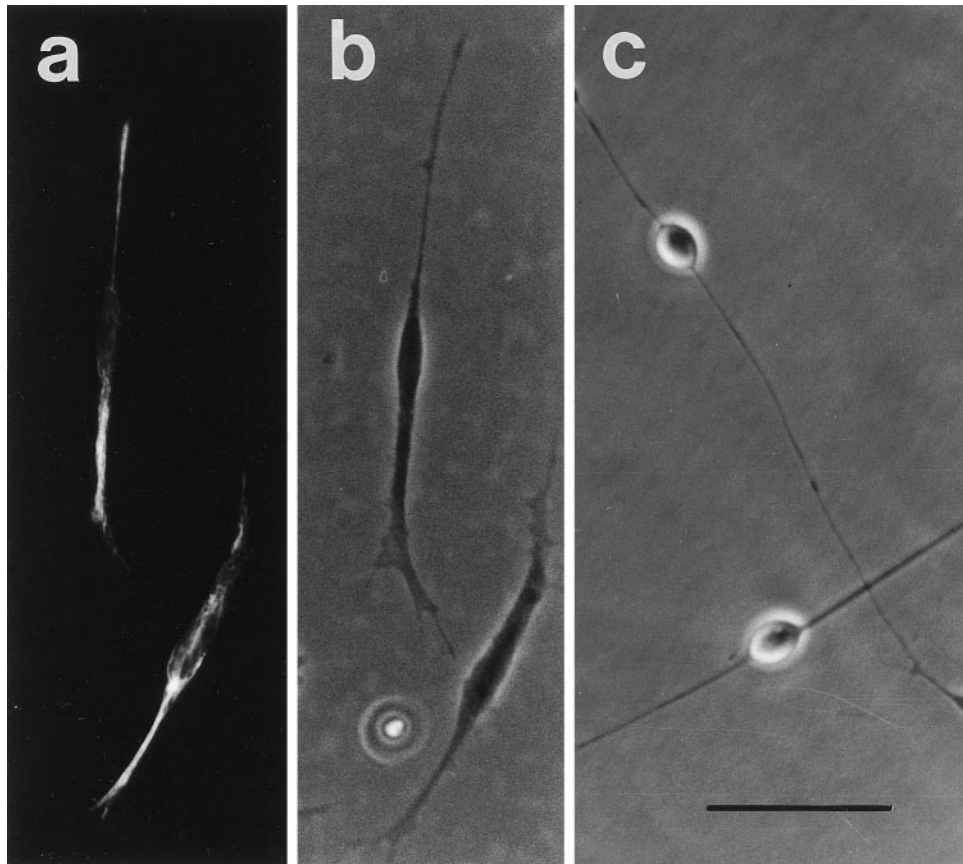
Figure 1. Photomicrographs of E11 trigeminal ganglion cultures showing the typical morphology of immature (*a*, *b*) and mature (*c*) early sensory neurons. The paired immunofluorescence (*a*) and phase-contrast (*b*) photomicrographs demonstrate that cells identified as immature neurons are stained with anti-neurofilament antiserum (Wright et al., 1992). Scale bar, 50 μm.

resulting from matings of *bcl-2*$^{+/-}$ mice. The trigeminal neurons of each embryo were cultured in triplicate in 60-mm-diameter poly-D-ornithine- and laminin-coated plastic tissue culture dishes at a density of 500–2000 neurons per dish in 5 ml of serum-free medium (Davies et al., 1993). Because many trigeminal neurons switch survival dependence from brain-derived neurotrophic factor (BDNF) to NGF between E11 and

E12 (Buchman and Davies, 1993; Paul and Davies, 1995), E11 neurons were grown with BDNF (5 ng/ml), and E12 neurons were grown with NGF (5 ng/ml).

Cohorts of morphologically immature neurons were defined in these cultures 18 hr after plating, and the fate of each neuron in these cohorts was followed for up to 3 d in culture. This was done by recording the
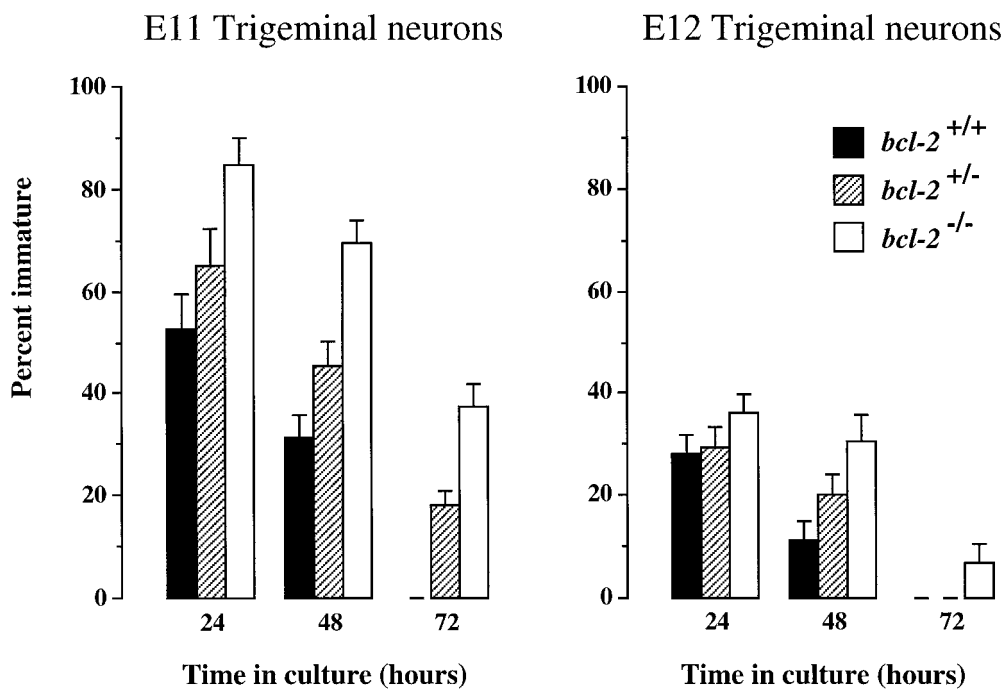

Figure 2. Bar charts showing the percentage of neurons that are immature in dissociated cultures of E11 and E12 trigeminal ganglia from *bcl-2*$^{-/-}$ and wild-type embryos after 24, 48, and 72 hr. The means + SE of the results of cultures established from 13 *bcl-2*$^{-/-}$, 26 *bcl-2*$^{+/-}$, and 14 *bcl-2*$^{+/+}$ embryos at E11 and 17 *bcl-2*$^{-/-}$, 29 *bcl-2*$^{+/-}$, and 18 *bcl-2*$^{+/+}$ embryos at E12 are shown.
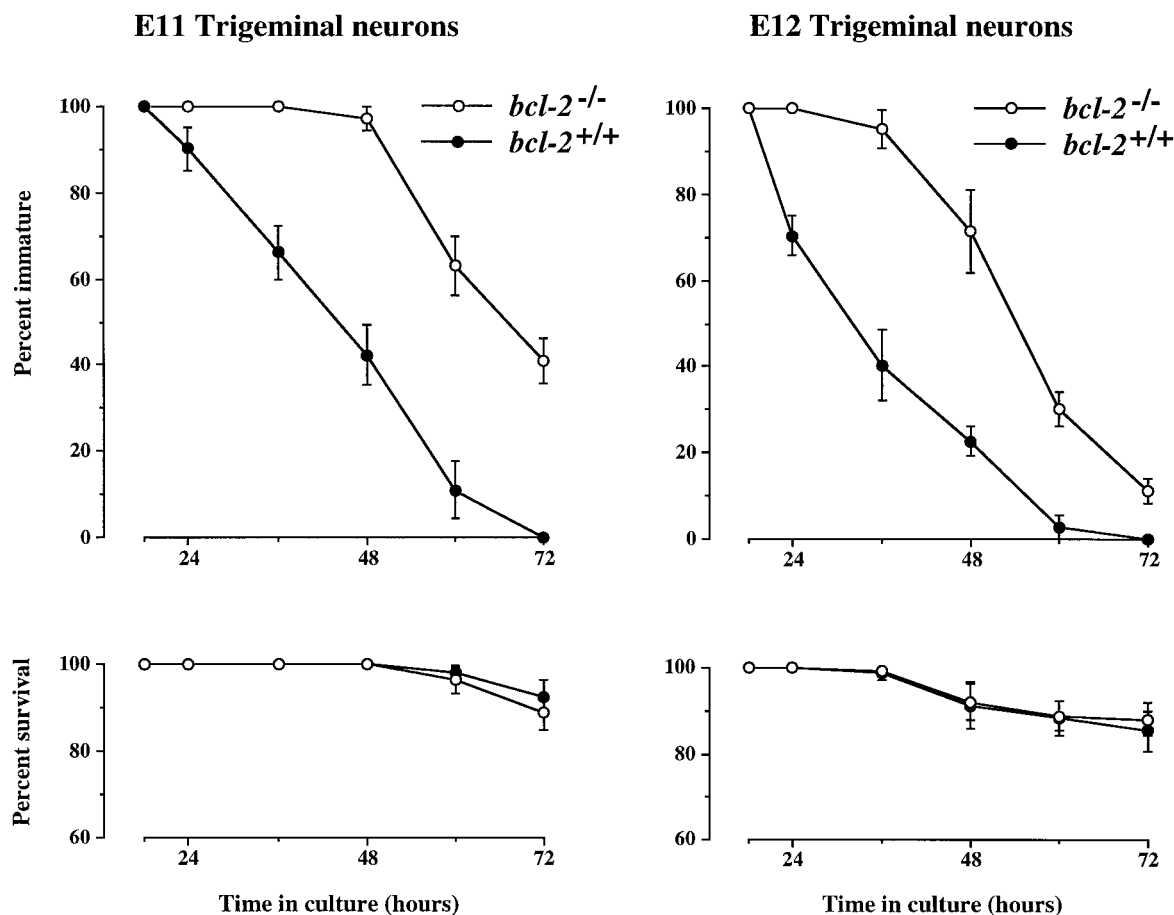
Figure 3. Graphs summarizing the results of neuron cohort experiments comparing the maturation and survival of trigeminal neurons from wild-type and *bcl-2* $^{-/-}$ embryos at E11 and E12. Cohorts of immature neurons were defined 18 hr after plating, and the number of immature neurons remaining in these cohorts (*top graphs*) and the number of neurons surviving in these cohorts (*bottom graphs*) are expressed as percentages of the initial cohort size at intervals up to 72 hr. The means ± SE of the results of cultures established from 24 *bcl-2* $^{-/-}$ and 23 *bcl-2* $^{+/+}$ embryos at E11 and 22 *bcl-2* $^{-/-}$ and 17 *bcl-2* $^{+/+}$ embryos at E12 are shown. The initial cohort size in each culture ranged from 20 to 60 neurons.

location 18 hr after plating of all immature neurons within a 9 × 9 mm grid inscribed on the undersurface of the culture dishes (Davies, 1989) and by monitoring at intervals if each of these neurons was still immature, had assumed a more mature morphology, or had degenerated.

Quantification of neurons in the trigeminal ganglion. E12, E13, and E14 mouse embryos in litters resulting from overnight matings of *bcl-2* $^{+/-}$ mice were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.3, and then embedded, serially sectioned at 8 μm, and stained with cresyl fast violet, and the total number of morphologically recognizable neurons in both trigeminal ganglia was quantified as described previously (Piñón et al., 1996). Neurons were identified by virtue of Nissl substance and large, round, pale-stained nuclei. Because of their small size, immature neurons cannot be distinguished from progenitor cells and glial cells in sections stained with cresyl fast violet and are therefore not included in these counts.

To identify all neurons (immature and mature) in the trigeminal ganglia of younger embryos, neurofilament staining was used. The heads of E12 embryos were fixed in Carnoy's fluid (60% ethanol, 30% chloroform, and 10% acetic acid) for 20 min before dehydration and wax embedding. Serial sections of the heads were cut at 8 μm, mounted on poly-L-lysine-coated slides, cleared in xylene, and dehydrated before quenching (10% methanol and 3% hydrogen peroxide in PBS). Nonspecific interactions were blocked using 10% horse serum in PBS with 0.4% Triton X-100. The sections were incubated in primary antibody (monoclonal anti-neurofilament 160; Sigma, St. Louis, MO) (used at 1:500) for 1 hr and then labeled using biotinylated secondary antibody (1:200), avidin, and biotinylated horseradish peroxidase macromolecular complex (Vectastain ABC kit; Vector Laboratories, Burlingame, CA). The substrate used for the peroxidase reaction was 1 mg/ml diaminobenzidine tetrachloride (Sigma). After staining, the sections were washed in tap water before rehydration and mounting. Estimation of neuronal number was performed as for cresyl fast violet-stained sections (Piñón et al., 1996). Here, neurons were identified as neurofilament-positive cells.

Measurement of specific mRNA levels. A quantitative reverse transcription-PCR (RT-PCR) technique was used to measure the levels of trkA and p75 mRNAs in total RNA extracted from pairs of trigeminal ganglia dissected from wild-type and *bcl-2* $^{-/-}$ mice (Wyatt and Davies, 1993). The reverse transcription and PCR reactions were calibrated by the inclusion of known amounts of cRNA competitor templates for each of the mRNAs in the reverse transcription reaction. The cRNA competitor templates were synthesized *in vitro* from cDNA competitor constructs. Details of the competitors, primers, and reaction conditions are provided elsewhere (Wyatt and Davies, 1993, 1995; Wyatt et al., 1997). Previous detailed comparisons of this method with quantitative Northern blotting have demonstrated its accuracy and reproducibility over a wide range of mRNA concentrations (Wyatt and Davies, 1993).

RESULTS

Neuronal maturation *in vitro*

Sensory neurons undergo a clearly recognized morphological change at an early stage in their development. After differentiating from progenitor cells, sensory neurons initially have small, spindle-shaped, phase-dark cell bodies. Subsequently, the cell bodies enlarge to become spherical and phase-bright (Wright et al., 1992). We refer to developing sensory neurons exhibiting these two morphologically distinct appearances as immature and mature early sensory neurons, respectively (Fig. 1). In the current
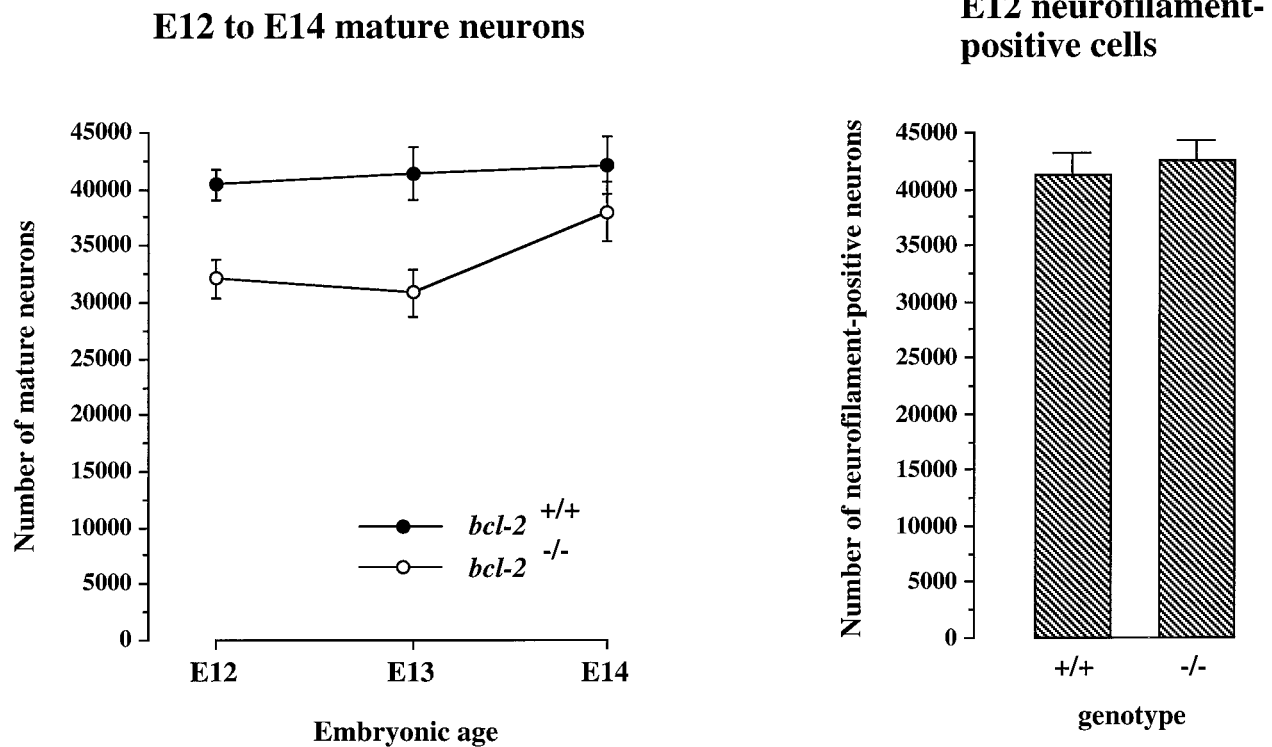
E12 to E14 mature neurons

E12 neurofilament-positive cells

Figure 4. Graph of the numbers of morphologically recognizable neurons in cresyl violet-stained sections of the trigeminal ganglia of wild-type and *bcl-2* $^{-/-}$ embryos at E12, E13, and E14 in the trigeminal ganglia of wild-type and *bcl-2* $^{-/-}$ embryos at E12, and bar chart of the numbers of neurons positively identified by neurofilament staining in the trigeminal ganglia of wild-type and *bcl-2* $^{-/-}$ embryos at E12. The means ± SE are shown (*n* = 53 embryos for mature neurons; *n* = 11 embryos for neurofilament-positive cells).

study, we have focused on this morphological change in the embryonic mouse trigeminal ganglion, a population of sensory neurons in which development is extensively characterized (Davies and Lumsden, 1984, 1986; Davies, 1987; Piñón et al., 1996; Wilkinson et al., 1996). The majority of neurons in this ganglion differentiate from proliferating progenitor cells between E9.5 and E13.5, and the first axons first emerge from the trigeminal ganglion at E9.5. The number of neurons peaks between E12 and E14 and decreases by 50% to reach a stable number by birth as a result of neuronal apoptosis that peaks at E14. We therefore studied the development of trigeminal neurons during the key early stages between E11 and E14 when most neurons are differentiating from progenitor cells and extending axons to their targets.

We observed that the proportion of immature neurons in dissociated cultures of early trigeminal ganglia from *bcl-2* $^{-/-}$ embryos was consistently higher than the proportion of immature neurons in cultures established from wild-type embryos of the same age (Fig. 2). In cultures established from trigeminal ganglia at E11 and E12 when many neurons are differentiating from progenitor cells and extending axons toward their targets *in vivo* (Davies and Lumsden, 1984; Wilkinson et al., 1996), the proportion of immature neurons was highest in cultures established from *bcl-2* $^{-/-}$ embryos at all time intervals examined (24, 48, and 72 hr). Furthermore, the proportion of immature neurons in cultures established from *bcl-2* $^{+/-}$ embryos was intermediate between that in cultures established from *bcl-2* $^{-/-}$ and wild-type embryos, suggesting a gene dosage effect. Similar results were obtained in E11 and E12 cultures of nodose ganglion neurons (data not shown).

An elevated number of immature neurons in dissociated cultures of early sensory ganglia from *bcl-2* $^{-/-}$ embryos could be attributable to a delay in the maturation of Bcl-2-deficient neurons, to differences in progenitor cell differentiation, or to differences in neuronal survival in these cultures. To directly address the question of whether Bcl-2-deficient early sensory neurons mature more slowly than wild-type neurons, we defined cohorts of immature neurons in cultures of E11 and E12 trigeminal neurons 18 hr after plating and followed the fate of each neuron in these cohorts for up to 3 d in culture (Fig. 3). These studies showed that Bcl-2-deficient neurons retain an immature morphology much longer than wild-type neurons; the maturation of Bcl-2-deficient neurons was delayed by 18–24 hr compared with wild-type neurons. These marked differences in the rate of neuronal maturation cannot be attributed to differences in the survival of Bcl-2-deficient and wild-type neurons, because very few neurons died in these cohorts throughout the period studied (Fig. 3). Because all data were obtained before the genotypes were ascertained, these data were not influenced by observer bias.

Development of early trigeminal neurons *in vivo*

To determine whether neuronal maturation is delayed in the trigeminal ganglia of *bcl-2* $^{-/-}$ embryos *in vivo*, we counted the morphologically recognizable mature neurons in the trigeminal ganglia of wild-type and *bcl-2* $^{-/-}$ embryos at several developmental stages. Neurogenesis in the mouse trigeminal ganglion and the recruitment of axons to the trigeminal nerve occur between E9.5 and E13.5 (Davies and Lumsden, 1984; Wilkinson et al., 1996). We began comparing the trigeminal ganglia of wild-type and *bcl-2* $^{-/-}$ embryos at E12 because large numbers of neurons
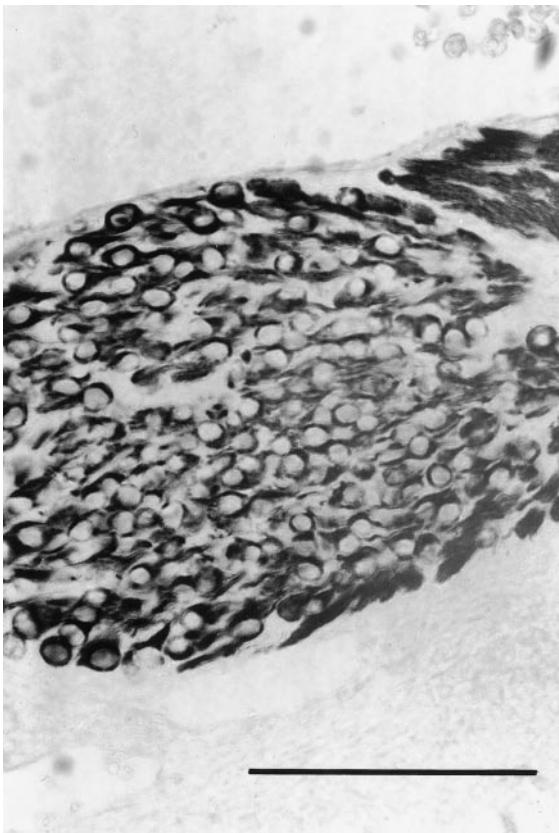
Figure 5. Photomicrograph of a section of the trigeminal of an E12 *bcl-2*$^{-/-}$ embryo stained for neurofilament protein. Scale bar, 100 μm.

become distinguishable by morphological criteria from other cell types at this stage (Davies and Lumsden, 1984). Because of their small size, immature neurons cannot be distinguished from progenitor cells and glial cells in cresyl fast violet-stained sections and are therefore not included in these counts. Figure 4 shows that there are significantly greater numbers of morphologically distinct neurons in the trigeminal ganglia of wild-type embryos at both E12 ($p = 0.0008$; $n = 17$, t test) and E13 ($p < 0.006$; $n = 18$, t test). By E14, however, the number of neurons of the trigeminal ganglia of *bcl-2*$^{-/-}$ embryos and wild-type embryos was not significantly different ($p > 0.1$; $n = 22$, t test). Because all data were obtained before the genotypes were ascertained, these data are not influenced by observer bias.

To ascertain whether the reduced number of morphologically identifiable neurons in the early trigeminal ganglia of *bcl-2*$^{-/-}$ embryos was attributable to a reduction in the number of neurons in the ganglion, we positively identified all neurons (mature and immature) in the trigeminal ganglion at E12 using neurofilament staining (Fig. 5). Estimates of neurofilament-positive cells in the ganglia of wild-type and *bcl-2*$^{-/-}$ embryos revealed no significant difference at this stage (Fig. 4). These observations suggest that the reduction in morphologically recognizable neurons in the trigeminal ganglia of *bcl-2*$^{-/-}$ embryos during the early stages of ganglion formation is not attributable to a reduction in neurons in the ganglion. These *in vivo* observations are consistent with the results of our *in vitro* studies that suggest that Bcl-2-deficient neurons retain an immature morphology longer *in vivo* than wild-type neurons.

Quantification of trkA and p75 mRNAs

To determine whether the delayed morphological maturation of early sensory neurons in *bcl-2*$^{-/-}$ embryos is a feature of a general delay in the program of development of these neurons, we used competitive RT-PCR to quantify the level of mRNAs encoding the NGF receptors trkA and p75. TrkA is a receptor tyrosine kinase that is essential for the survival response of neurons to NGF (Kaplan et al., 1991; Klein et al., 1991; Allsopp et al., 1993a), whereas p75 has several functions (Bothwell, 1995; Davies, 1997) including enhancing the survival response of trigeminal neurons to NGF (Davies et al., 1993). The levels of these mRNAs are known to increase rapidly during the early stages of trigeminal ganglion development and are expressed predominantly if not exclusively in the neurons of the ganglion in the embryo (Wyatt et al., 1990; Wyatt and Davies, 1993; Davies et al., 1995). Figure 6 shows that the levels of both mRNAs increased to the same extent in the trigeminal ganglia of wild-type and *bcl-2*$^{-/-}$ embryos between E12 and E14.

DISCUSSION

We have obtained compelling evidence for a novel role for Bcl-2 during the early stages of neuronal development that is distinct from its well recognized antiapoptotic function. Newly differentiated sensory neurons initially have small, spindle-shaped, phase-dark cell bodies that subsequently enlarge, becoming spherical and phase-bright. By tracking the morphology of individual neurons at intervals in cultures established from the early trigeminal ganglia of *bcl-2*$^{-/-}$ and wild-type embryos, we have demonstrated that this clearly recognizable morphological change is markedly delayed in Bcl-2-deficient neurons. This delay is not a consequence of a decrease in the viability of Bcl-2-deficient neurons, because early trigeminal neurons of *bcl-2*$^{-/-}$ and wild-type embryos survive equally well *in vitro*. Thus, the absence of Bcl-2 has a marked effect on the *in vitro* development of early sensory neurons that is unrelated to its role in regulating cell survival. Interestingly, later in development during the peak period of naturally occurring neuronal death, Bcl-2-deficient trigeminal neurons do die more rapidly than wild-type neurons in NGF-supplemented medium. This decrease in the *in vitro* viability of older embryonic Bcl-2-deficient trigeminal neurons is correlated with an increase in the number of dying neurons in the trigeminal ganglion at this stage *in vivo* and a reduction in the number of neurons in the ganglion before birth (Piñón et al., 1997). Reductions in the number of neurons in dorsal root and sympathetic ganglia and in motoneuron populations have also been reported in *bcl-2*$^{-/-}$ mice postnatally (Michaelidis et al., 1996).

Our *in vitro* observations of the delay in the morphological maturation of early Bcl-2-deficient sensory neurons were supported by an *in vivo* analysis of the trigeminal ganglia of *bcl-2*$^{-/-}$ and wild-type embryos. In cresyl fast violet-stained sections, there were fewer morphologically recognizable neurons in the trigeminal ganglia of *bcl-2*$^{-/-}$ embryos compared with wild-type embryos at E12 and E13, the stage when many neurons are differentiating from progenitor cells and starting to extend axons to their targets (Davies and Lumsden, 1984; Davies, 1987). Because of their small size, immature neurons are not distinguishable from progenitor cells and other non-neuronal cells in this histological analysis. The total neuronal complement of the trigeminal ganglion of *bcl-2*$^{-/-}$ embryos was, however, normal during this period of development, as revealed by counting the neurofilament-positive cells in the trigeminal ganglia of *bcl-2*$^{-/-}$ and wild-type embryos. The most parsimonious explanation for
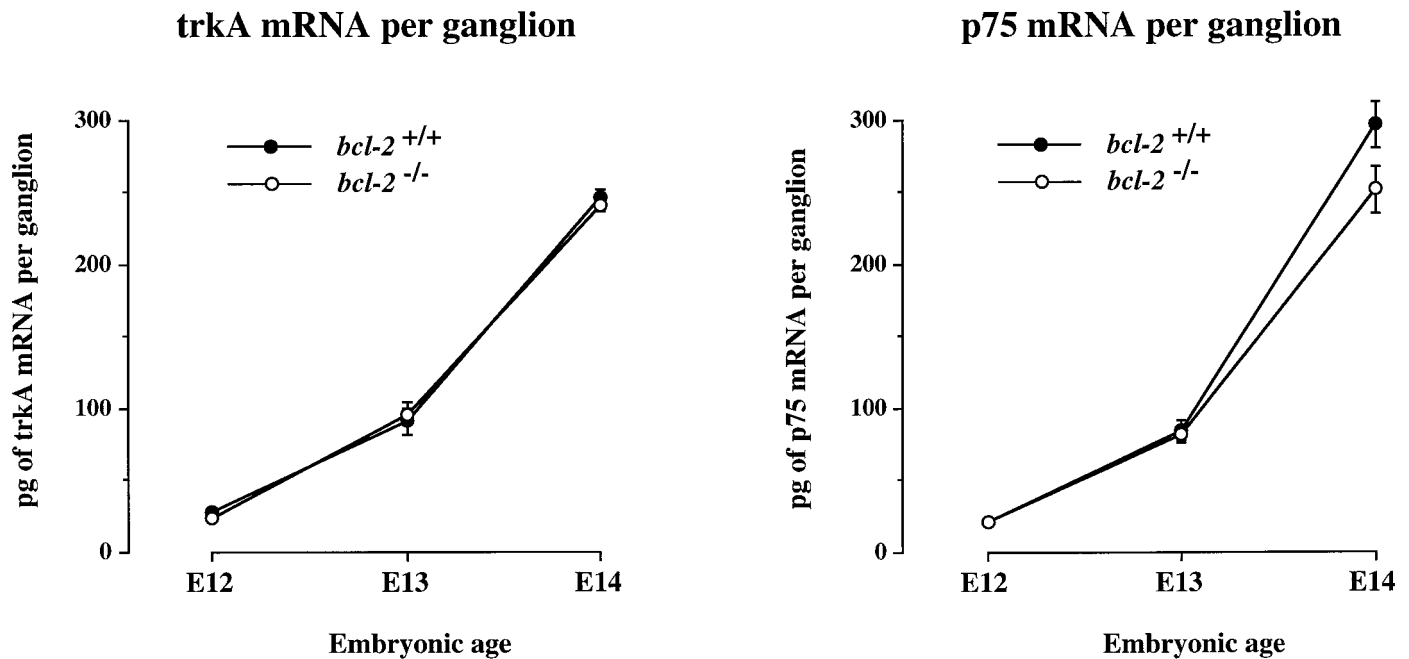
trkA mRNA per ganglion

p75 mRNA per ganglion

Figure 6. Graphs of the levels of trkA and p75 mRNA in the trigeminal ganglia of wild-type and *bcl-2* $^{-/-}$ embryos at E12, E13, and E14. The means ± SE are shown ($n = 42$ embryos).

these findings is that as *in vitro*, the morphological maturation of early sensory neurons is delayed *in vivo* in the absence of Bcl-2.

Of particular interest is our demonstration that the delay in the morphological maturation of early Bcl-2-deficient neurons is not a consequence of a general retardation of the development of *bcl-2* $^{-/-}$ embryos or of sensory neurons within these embryos. There was no apparent difference in the stage-specific external features (Theiler, 1972) of *bcl-2* $^{-/-}$ and wild-type embryos in each litter, and the time course and level of expression of p75 and trkA mRNAs were virtually identical in embryos of both genotypes. Because these mRNAs are expressed predominantly if not exclusively in the neurons of the embryonic trigeminal ganglion, and their levels increase markedly with the acquisition of the NGF survival response (Wyatt et al., 1990; Wyatt and Davies, 1993), these findings also reinforce our evidence based on estimates of the number of neurofilament-positive cells that the neuronal complement of the early trigeminal ganglion is similar in *bcl-2* $^{-/-}$ and wild-type embryos.

Several studies of the consequences of manipulating the expression of Bcl-2 in various cell lines and neurons have raised the possibility that Bcl-2 may have functions in addition to its well known antiapoptotic role. The differentiation of the PC12 pheochromocytoma cell line into neurons is enhanced by overexpressing Bcl-2. However, this effect is only observed when the cells are grown in serum-free medium (Batistatou et al., 1993). Midbrain-derived dopaminergic cell lines stably expressing Bcl-2 extend longer neurites than control-transfected cells but do not have increased expression of many neuron-specific proteins (Oh et al., 1996). Bcl-2 overexpression in a neural crest-derived cell line enhances the outgrowth of neurite-like processes and also increases the expression of neuron-specific enolase (Zhang et al., 1996). Several observations indicate that the level of Bcl-2 expression in mouse retinal ganglion cells (RGCs) influences the ability of their axons to regenerate into co-cultured tectal explants (Chen et al., 1997). At early fetal stages, RGCs normally express

high levels of Bcl-2 and are able to extend axons into co-cultured tectal tissue of the same age, whereas late fetal RGCs express low levels of Bcl-2 and are not able to grow axons into tectal tissue. The ability of early fetal RGCs to regenerate into tectal tissue is substantially reduced in retinal explants obtained from *bcl-2* $^{-/-}$ embryos, whereas late fetal and adult RGCs from transgenic mice overexpressing Bcl-2 in neurons are able to regenerate into tectal tissue. Recently, Bcl-2 expression has been shown to influence axonal growth rate during development. Early sensory neurons from *bcl-2* $^{-/-}$ mouse embryos extend axons more slowly *in vitro* than neurons from wild-type embryos of the same age (Hilton et al., 1997).

In summary, we have demonstrated a novel role for endogenously expressed Bcl-2 in sensory neurons shortly after they differentiate from progenitor cells. At this stage of development, Bcl-2 promotes an early morphological change in the neurons but does not influence certain other aspects of their development, such as the time course of NGF receptor expression. This action of Bcl-2 is unrelated to its well characterized antiapoptotic role and may provide an explanation for the widespread expression of Bcl-2 in embryonic nervous system during the earliest stages of neuronal development (Abe et al., 1993; Merry et al., 1994), whereas abnormal degeneration of neurons in Bcl-2-deficient mice is not observed until the late fetal and postnatal periods (Michaelidis et al., 1996; Piñón et al., 1997).

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