Bcl-2 is required for cranial sensory neuron survival at defined stages of embryonic development

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SUMMARY

To ascertain the role of endogenous Bcl-2 in maintaining the survival of developing neurons and modulating their responses to neurotrophins, we compared the in vitro and in vivo survival of cranial sensory neurons of wild-type and *bcl-2* null mouse embryos. At the peak of naturally occurring neuronal death in the trigeminal ganglion at E14, trigeminal neurons from *bcl-2*−/− embryos initially survived in culture in response to NGF but were not sustained as well as neurons from wild-type embryos. At the end of the period of naturally occurring neuronal death at E18, Bcl-2-deficient trigeminal neurons survived with NGF as well as wild-type neurons. At E14 in vivo, the number of trigeminal neurons undergoing apoptosis was significantly greater in *bcl-2*−/−

embryos, and there were significantly fewer neurons in the trigeminal ganglia of *bcl-2*−/− embryos at E16 and E18. Similar age-related changes in the responses of nodose ganglion neurons to BDNF were observed in cultures established from *bcl-2*−/− and wild-type embryos between E14 and E18. These results suggest that endogenous Bcl-2 is required for the sustained survival response of a subset of cranial sensory neurons to neurotrophins at particular stages of embryonic development and show that its absence leads to reduced numbers of these neurons in vivo.

Key words: Bcl-2, neuron survival, sensory neuron, cranial neuron, cell death, mouse

INTRODUCTION

Bcl-2 is the founder member of a family of vertebrate cytoplasmic proteins related to ced-9, an inhibitor of programmed cell death in the nematode *C. elegans* (Hengartner and Horvitz, 1994). Bcl-2 is widely expressed in different tissues and considerable evidence suggests that it plays an important role in regulating cell survival in the immune system. Whereas mice with targeted null mutations in the *bcl-2* gene have markedly reduced numbers of B and T cells due to increased apoptosis (Nakayama et al., 1993, 1994; Veis et al., 1993), 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).

Experimental overexpression of Bcl-2 in developing neurons has shown that Bcl-2 is also capable of influencing neuronal survival. Microinjection of a Bcl-2 expression vector into cultured sympathetic and sensory neurons prevents their death following neurotrophin deprivation (Allsopp et al., 1993; Garcia et al., 1992), and mice expressing a *bcl-2* transgene under the control of a neuron-specific enolase promoter have enlarged brains and increased numbers of neurons in several regions (Martinou et al., 1994). There is some evidence from in vitro experiments that the expression of endogenous Bcl-2 is able to influence the survival of developing neurons. Antisense *bcl-2* RNA markedly and selectively reduces the survival response of embryonic proprioceptive neurons to BDNF but does not affect their response to CNTF or GPA (Allsopp et al., 1995). Sympa-

thetic neurons from neonatal mice that have a null mutation in the *bcl-2* gene die more rapidly following NGF deprivation in vitro than wild-type neurons (Greenlund et al., 1995).

Although Bcl-2 is capable of influencing neuronal survival in vitro and its overexpression can prevent naturally occurring neuronal death in vivo, it is not clear to what extent Bcl-2 plays a role in regulating neuronal survival in the embryo. In the developing nervous system, Bcl-2 is expressed in proliferating neuroepithelial cells of ventricular zones and in postmitotic cells of several regions including the cortical plate, cerebellum, hippocampus and spinal cord (Merry et al., 1994). Bcl-2 expression decreases in CNS neurons postnatally, but is retained in sensory and sympathetic neurons throughout life. Although this pattern of expression is consistent with a role for Bcl-2 in regulating neuronal survival during development and sustaining the survival of some neurons in the adult, *bcl-2*−/− mice appear to have a grossly normal nervous system and do not exhibit any obvious neurological abnormalities (Nakayama et al., 1993, 1994; Veis et al., 1993). Statistically significant deficiencies in sensory, motor and sympathetic neurons have, however, recently been described in postnatal *bcl-2* null mice (Michaelidis et al., 1996).

To clarify the role of Bcl-2 in regulating neuronal survival during embryonic development, we have carried out in vivo and in vitro studies of the survival of sensory neurons of mouse embryos that have a null mutation in the *bcl-2* gene. We have focused on two well-characterised populations of cranial sensory neurons that are supported by different neurotrophins during the

period of naturally occurring neuronal death. The majority of mouse trigeminal ganglion neurons are supported by NGF in culture from E12 onwards and depend on NGF derived from their peripheral targets for survival during the period of naturally occurring neuronal death in vivo (Buchman and Davies, 1993; Davies et al., 1987; Piñón et al., 1996). The majority of nodose ganglion neurons are supported by BDNF in culture from E12 onward and depend on BDNF for survival in vivo during embryonic development (Davies et al., 1993; Ernfors et al., 1994; Jones et al., 1994). The total number of neurons in the trigeminal ganglion peaks between E13 and E14 and falls by half by birth as a result of naturally occurring neuronal death (Davies and Lumsden, 1984) with a peak of apoptosing neurons in the ganglion at E14 (Piñón et al., 1996). Our results show that the survival response of Bcl-2-deficient trigeminal and nodose neurons to neurotrophins is less sustained than that of wild-type neurons during the early fetal period, and that there is a significant decrease in the number of neurons in the trigeminal ganglia of *bcl-2* null mice during embryonic development.

MATERIALS AND METHODS

Experimental animals

Embryos were obtained from overnight matings of bcl-2$^{+/-}$ mice (generous gift of Dennis Loh). Pregnant females were killed by cervical dislocation at the required stage of gestation and the precise stage of development of the embryos was determined by the criteria of Theiler (1972). The genotypes of the embryos were determined by a PCR-based technique using DNA isolated from embryonic tissues.

Neuron cultures

Separate dissociated cultures of trigeminal and nodose ganglion neurons were set up from each embryo (Davies et al., 1995). The neurons were grown in 35 mm diameter plastic tissue culture wells (Nunc) that had been previously coated with polyornithine (0.5 mg/ml, overnight) and laminin (20 µg/ml for 4 hours). The neurons were plated at a density of approximately 500-2,000 neurons per dish in 2 ml of Ham's F14 supplemented with 2 mM glutamine, 0.35% bovine serum albumin (Pathocyte-4, ICN), 60 ng/ml progesterone, 16 µg/ml putrescine, 400 ng/ml L-thyroxine, 38 ng/ml sodium selenite, 340 ng/ml tri-iodo-thyronine, 60 µg/ml penicillin and 100 µg/ml streptomycin. The cultures were incubated at 37°C in a 5% CO_2 incubator.

The number of attached neurons within a 12×12 mm square in the centre of each dish was counted 6 hours after plating; the mean of these counts was taken as the initial number of neurons the experiment. The number of surviving neurons was counted at intervals up to 72 hours in the same 12×12 mm area in each dish and is expressed as a percentage of the number of neurons counted at 6 hours. In each experiment, triplicate cultures were set up for all conditions. Control cultures did not receive neurotrophins, otherwise purified recombinant neurotrophins made in bacteria were added to the cultures at the time of plating (gifts of John Winslow and Gene Burton of Genentech, Inc.).

Quantification of numbers of living and dying neurons in the trigeminal ganglion

Embryos were fixed by immersion in 4% paraformaldehyde in 0.1 M phosphate buffer pH 7.3 following removal of the brain. After fixation for at least a week, the embryos were dehydrated and embedded in paraffin wax. E16 and E18 embryos were first decalcified with 1 M EDTA. Coronal serial sections of the head were cut at 8 µm. These were mounted on either gelatinised slides or polylysine-coated slides and were stained with cresyl fast violet.

To evaluate the extent of cell death, all pyknotic nuclei were counted at 400× magnification in every fifth section along the entire rostrocaudal extent of the trigeminal ganglion. Estimates of the total

number of pyknotic nuclei in each ganglion were obtained by multiplying the sum of these counts by five. In all cases, the sections were coded prior to counting to avoid any observer bias.

To estimate the total number of neurons in the trigeminal ganglion, the ganglion profile of every 10th section was drawn using a drawing tube at 100× magnification and its cross-sectional area was measured using an Image Processing and Analysis Program (NIH Image). The average neuron density in each of these sections was quantified at 1,000× magnification using a 0.01 mm^2 grid such that at least 25% of the ganglion in each section was sampled. Neurons were identified by virtue of the Nissl substance and their large, round, pale-stained nuclei (Konigsmark, 1970). The total number of neurons in each section was calculated from the section area and average neuron density in each section. The total number of neurons in the ganglion was estimated by adding these numbers and multiplying by 10. Correction for split nucleoli was not made as these do not appreciably affect the neuronal estimate (Jones, 1937).

RESULTS

Response of Bcl-2-deficient sensory neurons to neurotrophins in vitro

Mice that were heterozygous for a *bcl-2* null mutation (Nakayama et al., 1993, 1994) were crossed to obtain *bcl-2$^{-/-}$*, *bcl-2$^{+/-}$* and *bcl-2$^{+/+}$* embryos. Separate, low-density, dissociated cultures were established from the trigeminal and nodose ganglia of each embryo and the survival of the neurons growing with and without neurotrophins was monitored for 3 days in culture. The genotypes of the embryos were subsequently determined using DNA extracted from embryonic tissues.

(i) Trigeminal neurons

Dissociated cultures of trigeminal ganglion neurons were established from embryos at E14, E16 and E18. The neurons were grown with or with out NGF for 72 hours and the number of surviving neurons was counted at intervals during this time. At E14, which is at the peak of naturally occurring neuronal death in the trigeminal ganglion (Piñón et al., 1996), about half of the initial number of neurons from wild-type embryos were still surviving in the presence of NGF after 72 hours incubation (Fig. 1). In marked contrast, neurons from *bcl-2$^{-/-}$* embryos died much more rapidly in the presence of NGF and all were dead by 48 hours. Neurons from *bcl-2$^{+/-}$* embryos died at an intermediate rate in the presence of NGF and all were dead by 72 hours. Although Bcl-2-deficient neurons died rapidly in the presence of NGF, they survived better than neurons grown without NGF in the culture medium. In these control cultures, all neurons were dead by 24 hours, and there was no difference in the rate of neuronal death between neurons from wild-type and Bcl-2-deficient embryos (data not shown). These results suggest that endogenous Bcl-2 expression is required for the sustained survival response of trigeminal neurons to NGF at E14.

In E16 cultures, the *bcl-2* null mutation had a much smaller effect on the NGF survival response (Fig. 1). Although there were significantly fewer Bcl-2-deficient neurons surviving with NGF after 24, 48 and 72 hours incubation compared with wild-type neurons ($P<0.05$, *t*-tests), there was on average only a 20% reduction in the number of surviving neurons at these time points. Both wild-type and Bcl-2-deficient neurons died rapidly in the absence of NGF at this stage and the *bcl-2* null mutation did not affect the rate of neuronal death in these control cultures (data not shown).
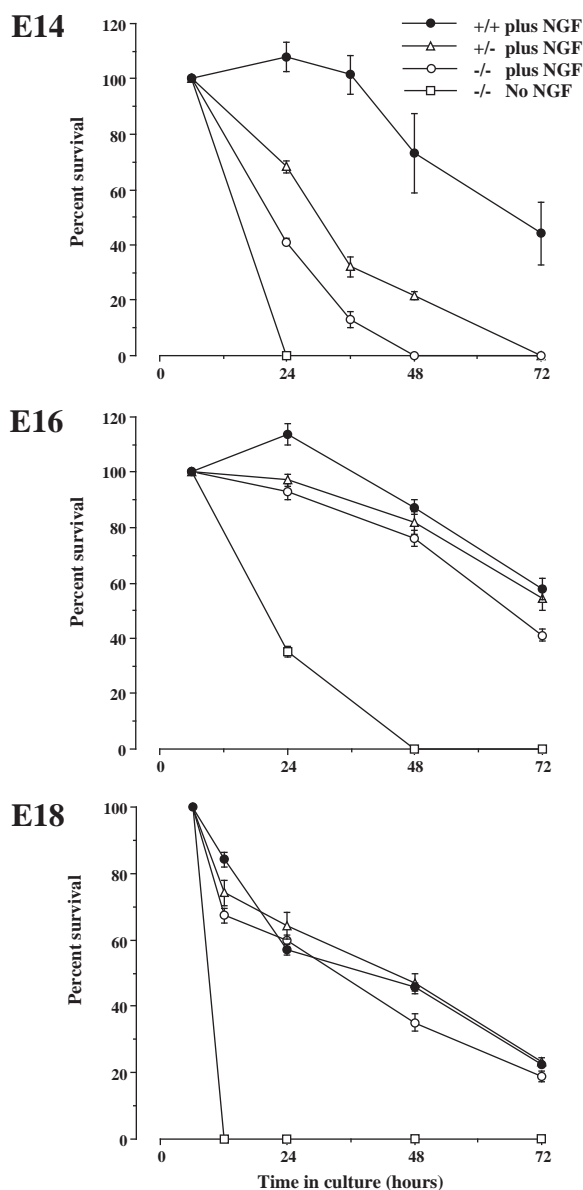
Fig. 1. Graphs of the percentage survival of E14, E16 and E18 trigeminal ganglion neurons from *bcl-2*$^{-/-}$, *bcl-2*$^{+/-}$ and *bcl-2*$^{+/+}$ embryos grown for 72 hours in culture with 5 ng/ml NGF in the culture medium. The survival of neurons from *bcl-2*$^{-/-}$ embryos growing without neurotrophins in the culture medium (control cultures) is also shown. The number of attached neurons in these cultures was counted after 6 hours incubation and the number of neurons survival at later times are expressed as percentages of these initial counts. The means and s.e.m.s (standard errors of the means) are shown. Between 11 and 25 embryos of each genotype were used at E14, between 6 and 13 embryos of each genotype at E16 and between 5 and 14 embryos of each genotype at E18. Triplicate control and NGF-supplemented dishes were set up for each embryo.

By E18, which is near the end of the period of naturally occurring neuronal death in the trigeminal ganglion (Davies and Lumsden, 1984; Piñón et al., 1996), there were no significant differences between the numbers of wild-type and Bcl-2-deficient neurons surviving in the presence of NGF (Fig. 1). These results suggest that, by the end of the period of naturally

occurring neuronal death, Bcl-2 is not required for a sustained survival response of trigeminal ganglion neurons to NGF.

(ii) Nodose neurons

Very similar trends were observed in the BDNF survival responses of nodose neurons from *bcl-2*$^{-/-}$, *bcl-2*$^{+/-}$ and *bcl-2*$^{+/+}$ embryos at E14, E16 and E18 as were seen in the NGF survival responses of trigeminal neurons over this period of development. In E14 cultures, over 40% of nodose neurons from wild-type embryos were still surviving in the presence of BDNF after 72 hours incubation, whereas neurons from *bcl-2*$^{-/-}$ embryos died much more rapidly in the presence of BDNF and all were dead by 48 hours (Fig. 2). Neurons from *bcl-2*$^{+/-}$ embryos died at an intermediate rate in the presence of BDNF and less than 10% were surviving by 72 hours. Although Bcl-2-deficient neurons died rapidly in the presence of BDNF, they survived better than control neurons grown without BDNF. In these control cultures, all neurons were dead by 24 hours, and there was no difference in the rate of neuronal death between neurons from wild-type and Bcl-2-deficient embryos (data not shown). Like the survival response of E14 trigeminal neurons to NGF, these results suggest that endogenous Bcl-2 expression is required for the sustained survival response of early fetal nodose neurons to BDNF.

In E16 cultures, the *bcl-2* null mutation had a less dramatic effect on the response of nodose neurons to BDNF (Fig. 2). Although there were 10 to 15% fewer neurons from *bcl-2* null mice surviving with BDNF compared with the number of neurons from wild-type mice at 24 and 48 hours, by 72 hours there was a greater than 3-fold difference in the numbers of neurons from *bcl-2*$^{-/-}$ and *bcl-2*$^{+/+}$ embryos surviving with BDNF. At all time points, these differences were statistically significant ($P<0.05$). By E18, there were no clear differences between the numbers of neurons from *bcl-2*$^{-/-}$ and *bcl-2*$^{+/+}$ embryos surviving in the presence of BDNF whereas, in the absence of BDNF, all neurons died rapidly (Fig. 2). As with the survival response of developing trigeminal neurons to NGF, these results suggest that, by the late fetal period, Bcl-2 is not required for a sustained survival response of nodose neurons to BDNF.

Bcl-2-deficient sensory neurons have normal neurotrophin dose responses

Although Bcl-2 expression was required for the sustained survival response of early fetal trigeminal and nodose neurons to NGF and BDNF, respectively, it did not appear to affect the sensitivity of these neurons to these neurotrophins. Fig. 3 shows the NGF dose responses of E14 trigeminal neurons from *bcl-2*$^{-/-}$, *bcl-2*$^{+/-}$ and *bcl-2*$^{+/+}$ embryos scored 12 hours after plating before the increased rate of loss of Bcl-2-deficient neurons becomes apparent. This graph shows that there was no obvious difference in the dose responses of these neurons to NGF; the ED$_{50}$ for all three genotypes was approximately 10 pg/ml. Likewise, the dose responses of E14 nodose neurons from *bcl-2*$^{-/-}$, *bcl-2*$^{+/-}$ and *bcl-2*$^{+/+}$ embryos were similar (Fig. 3). Although more neurons had died in cultures of Bcl-2-deficient nodose neurons than wild-type neurons by 12 hours (when survival was quantified), there were no obvious differences in the sensitivity of the neurons to BDNF at concentrations up to the peak survival at 80 pg/ml. The ED$_{50}$ was approximately 5 pg/ml in cultures established from all three genotypes. However, between 80 pg/ml and 400 pg/ml, there was a decrease in the survival of Bcl-2-deficient neurons

whereas the survival of wild-type neurons remained similar at these concentrations. Although by 24 hours, the level of survival of Bcl-2-deficient neurons was similar at these concentrations (data not shown).

bcl-2 null embryos have reduced numbers of sensory neurons

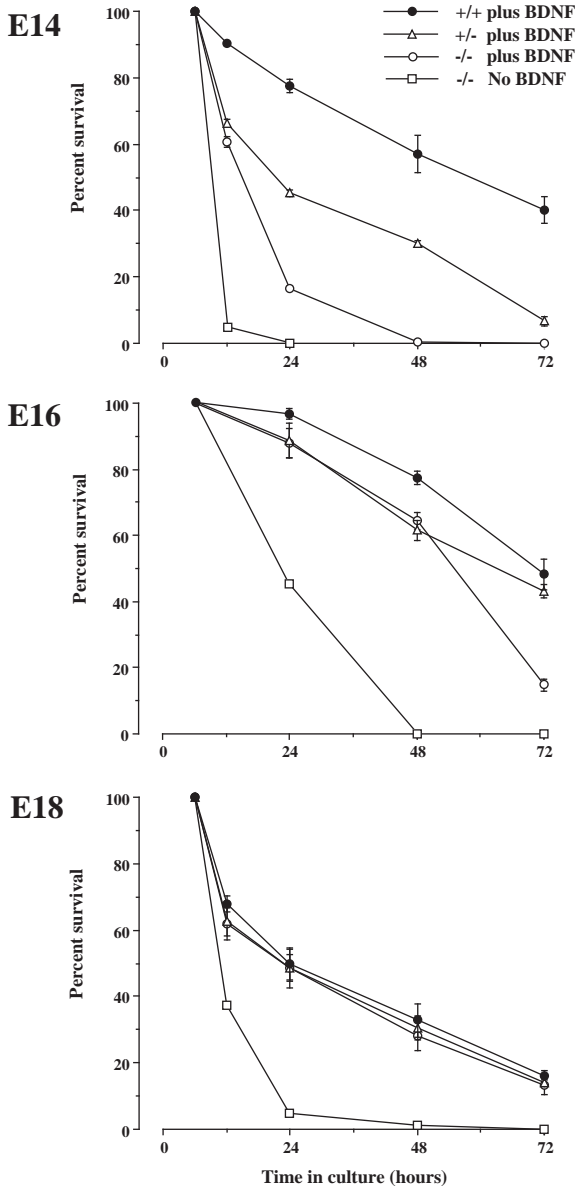To determine if the short-lived survival responses of Bcl-2-

Fig. 2. Graphs of the percent survival of E14, E16 and E18 nodose ganglion neurons from *bcl-2*⁻/⁻, *bcl-2*⁺/⁻ and *bcl-2*⁺/⁺ embryos grown for 72 hours in culture with 5 ng/ml BDNF in the culture medium. The survival of neurons from *bcl-2*⁻/⁻embryos growing without neurotrophins in the culture medium is also shown. The number of attached neurons in these cultures was counted after 6 hours incubation, and the number of neurons survival at later times are expressed as percentages of these initial counts. The means and s.e.m.s are shown. Between 11 and 25 embryos of each genotype were used at E14, between 6 and 13 embryos of each genotype at E16 and between 5 and 14 embryos of each genotype at E18. Triplicate control and BDNF-supplemented dishes were set up for each embryo.

deficient neurons to neurotrophins in vitro are physiologically relevant, we compared the number of neurons undergoing apoptosis and the total number of neurons in the trigeminal ganglia of wild-type and *bcl-2* mutant embryos at E14 and at later stages during the period of naturally occurring neuronal death (Davies and Lumsden, 1984; Piñón et al., 1996). Embryos were prepared for routine histology and the number of neurons with pyknotic nuclei and the total number of neurons counted in serial sections of the trigeminal ganglia. All histology slides were coded so that these estimates were made without knowledge of the genotype.

The estimates of the total numbers of neurons in the trigeminal ganglia of wild-type embryos over the age range studied were very similar to previously reported estimates (Davies and Lumsden, 1984; Piñón et al., 1996), showing an approximate 50% decrease between E14 and E18 (Fig. 4). Although there were fewer neurons in the trigeminal ganglia of *bcl-2*⁻/⁻ embryos compared with wild-type embryos at E14, this difference was not statistically significant ($P>0.1$, t-test, $n=22$). There were, however, statistically significant reductions of approximately 25% in the number of neurons in the trigeminal
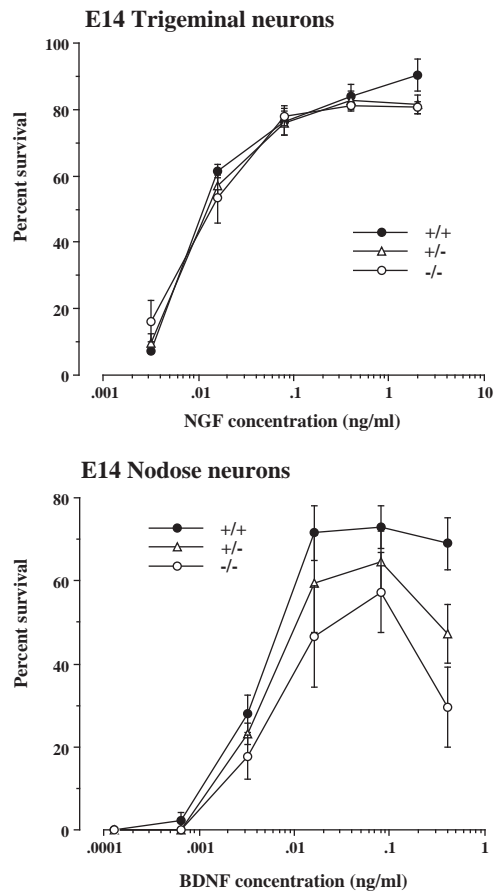

Fig. 3. Dose response of E14 trigeminal ganglion neurons and E14 nodose ganglion neurons from *bcl-2*⁻/⁻, *bcl-2*⁺/⁻ and *bcl-2*⁺/⁺ embryos to a range of concentrations of NGF and BDNF, respectively. The numbers of neurons surviving in these cultures after 12 hours incubation are expressed as a percentage of the number of neurons plated. The means and s.e.m.s of cultures from at least four separate cultures of neurons from each genotype, each set up in triplicate, are shown.
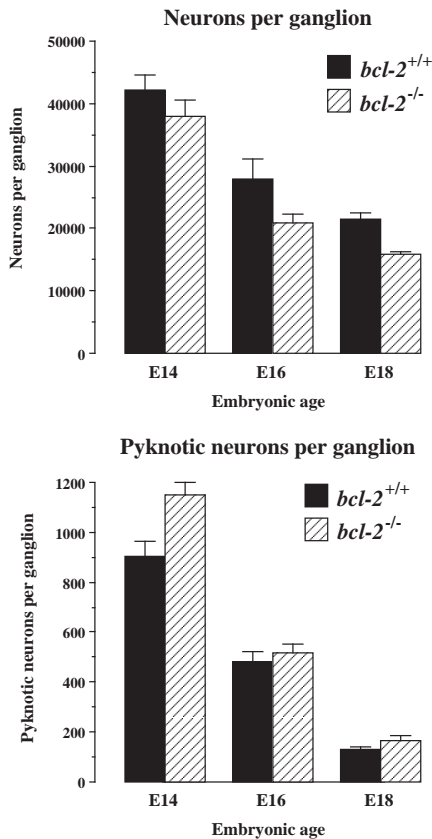
Neurons per ganglion

Pyknotic neurons per ganglion

Fig. 4. Numbers of neurons and pyknotic nuclei in the trigeminal ganglia of *bcl-2⁻/⁻* and *bcl-2⁺/⁺* embryos at E14, E16 and E18. The means and s.e.m.s of counts from 10 to 16 ganglia of each genotype at each age are shown.

ganglia of *bcl-2⁻/⁻* embryos compared with wild-type embryos at E16 (*P*<0.05, *t*-test, *n*=28) and E18 (*P*<0.001, *t*-test, *n*=18). There was also a significant increase in the number of pyknotic nuclei in the trigeminal ganglia of *bcl-2⁻/⁻* embryos compared with wild-type embryos at E14 (28% increase, *P*<0.01, *t*-test, *n*=18). Although there were slightly more pyknotic neurons in the trigeminal ganglia of *bcl-2⁻/⁻* embryos compared with wild-type embryos at E16 and E18, these differences were not statistically significant. These results suggest that, in the absence of Bcl-2, an proportion of trigeminal neurons undergo apoptosis at E14 and this is reflected in a reduction in the total number of neurons in the ganglion at later ages.

DISCUSSION

We have shown that the cranial sensory neurons of the trigeminal and nodose ganglia of *bcl-2⁻/⁻* embryos at E14 do not have a sustained in vitro survival response to their respective neurotrophins, NGF and BDNF. Although Bcl-2-deficient trigeminal and nodose neurons initially display a survival response to neurotrophins that has similar dose-response characteristics as wild-type neurons, these neurons die rapidly in the presence of neurotrophins compared with wild-type neurons. However, at later developmental stages, the difference in the survival response between wild-type and Bcl-2-deficient neurons is much less pronounced and, by E18, wild-type and Bcl-2-deficient trigeminal and nodose neurons survive equally well with NGF and BDNF,

respectively. It has previously been shown that the number of neurons in the trigeminal ganglion peaks between E13 and E14 and falls by half to reach a stable number by birth as a result of naturally occurring neuronal death (Davies and Lumsden, 1984). Accordingly, the number of apoptotic neurons in the trigeminal ganglion peaks at E14 and declines to negligible levels by birth (Piñón et al., 1996). Our in vitro findings therefore suggest that Bcl-2 expression is required for the sustained survival response of at least some cranial sensory neurons to neurotrophins during the early phase of naturally occurring neuronal death.

Our finding that the survival of Bcl-2-deficient cranial sensory neurons to neurotrophins is short lived during the early stages of naturally occurring neuronal death accords with our previous demonstration that microinjection of antisense *bcl-2* expression constructs into E10 chicken trigeminal mesencephalic nucleus (TMN) neurons causes a substantial and specific reduction in the number of these neurons surviving with BDNF (Allsopp et al., 1995). Naturally occurring neuronal death takes place in the chicken TMN between E9 and E14 (Rogers and Cowen, 1974), thus reduced Bcl-2 expression appears to compromise the neurotrophin survival response during the early stages of naturally occurring neuronal death. Interestingly, earlier in development, shortly after differentiating from progenitor cells, sensory neurons from *bcl-2⁻/⁻* embryos and wild-type embryos survive equally well in culture (G. Middleton and A. M. Davies, unpublished observations).

Although we have shown that endogenous Bcl-2 expression is apparently not required for the sustained neurotrophin survival response of mouse cranial sensory neurons at the end of the period of naturally occurring neuronal death, injection of a Bcl-2 expression vector into the trigeminal neurons of E18 and newborn wild-type mice is still able to prevent their death following NGF deprivation (J. Adu and A. M. Davies, unpublished observations). Likewise, DRG neurons isolated from transgenic mice expressing Bcl-2 under the control of the neuron-specific enolase promoter showed enhanced survival in the absence of NGF (Farlie et al., 1995).

To ascertain the physiological significance of our in vitro observations, we compared the numbers of surviving and dying neurons in the trigeminal ganglia of wild-type and *bcl-2* mutant embryos during the period of naturally occurring neuronal death. In contrast to initial reports that the nervous system of *bcl-2⁻/⁻* animals is grossly normal (Nakayama et al., 1993, 1994; Veis et al., 1993), we have shown that there is a significant increase in the number of dying neurons in the trigeminal ganglia of *bcl-2⁻/⁻* embryos at E14 and this is reflected in a 25% reduction in the total number of neurons in the trigeminal ganglia of Bcl-2-deficient embryos at E16 and E18. Our findings therefore suggest that Bcl-2 expression is necessary for sustaining the survival of a proportion of trigeminal ganglion neurons to NGF during the early stages of naturally occurring neuronal death in the trigeminal ganglion. These in vivo findings are consistent with our demonstration that the in vitro survival response of trigeminal neurons to NGF is impaired during the early stages of naturally occurring neuronal death. We have therefore demonstrated the existence of specific neuronal deficiencies in *bcl-2⁻/⁻* mice that are consistent with the impaired survival response of Bcl-2-deficient neurons to neurotrophins in vitro and provide evidence for a physiological role for Bcl-2 in sustaining the survival of neurons during embryonic development.

Although Bcl-2 expression is only required for the survival

of a subset of trigeminal neurons during development, the related protein Bcl-x (Boise et al., 1993) has been shown to play a prominent role in regulating the survival embryonic sensory neurons. $bcl-x^{-/-}$ mice die in utero at E13 with extensive neuronal apoptosis in the central nervous system and dorsal root ganglia (Motoyama et al., 1995), suggesting that the survival of many neurons is dependent on Bcl-x expression during the earliest stages of their development. Because Bcl-x expression is retained in dorsal root and cranial sensory ganglia in postnatal and adult mice (Krajewski et al., 1994; Gillardon et al., 1996), it is possible that Bcl-x is also required for the survival of many sensory neurons at later stages of embryonic development and in the adult.

Bcl-2 expression is also retained in sensory, sympathetic and motor neurons throughout life (Merry et al., 1994), and a recent study of *bcl-2* null mice has revealed the appearance of statistically significant deficiencies in these populations of neurons during the postnatal period (Michaelidis et al., 1996). Between P3 and P9, the number of motoneurons in the facial nucleus of $bcl-2^{-/-}$ falls to 71% of that in wild-type mice and between P3 and P10 the number of sympathetic neurons in the superior cervical sympathetic ganglion falls to 60% of normal. There is also a significant reduction in the number of DRG neurons, but this does not become apparent until P44. Although we have not investigated if further decreases in the neuronal complement of cranial sensory ganglia take place postnatally in $bcl-2^{-/-}$ mice, we have shown that Bcl-2 expression is required for maintaining the correct number of neurons in cranial sensory ganglia during the period of naturally occurring neuronal death and that this agrees with the impaired survival response of these neurons to neurotrophins during this critical period of embryogenesis.

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