

## Populations of NGF-dependent neurones differ in their requirement for BAX to undergo apoptosis in the absence of NGF/TrkA signalling in vivo

Gayle Middleton and Alun M. Davies\*

Department of Preclinical Veterinary Sciences, Royal (Dick) School of Veterinary Studies, Summerhall Square, Edinburgh EH9 1QH, UK

\*Author for correspondence (e-mail: alun.davies@ed.ac.uk)

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### SUMMARY

Reports that apoptosis within populations of neurotrophin-dependent neurones is virtually eliminated in BAX-deficient mice and that BAX-deficient neurones survive indefinitely in culture without neurotrophins have led to the view that BAX is required for the death of neurotrophin-deprived neurones. To further examine this assertion in vivo, we have studied two populations of NGF-dependent neurones during the period of naturally occurring neuronal death in mice that lack BAX, NGF or the NGF receptor TrkA, alone and in combination. In the superior cervical ganglion (SCG), naturally occurring neuronal death and the massive loss of neurones that took place in the absence of NGF or TrkA were completely prevented by elimination of BAX. However, in the trigeminal ganglion, naturally occurring neuronal death was only partly abrogated by the elimination of BAX, and although the massive neuronal death that took place in this ganglion in the absence of NGF

or TrkA was initially delayed in embryos lacking BAX, this subsequently occurred unabated. Accordingly, BAX-deficient neurones survived in defined without NGF whereas BAX-deficient trigeminal neurones died in the absence of NGF. These results indicate that whereas BAX is required for the death of SCG neurones during normal development and when these neurones are deprived of NGF/TrkA signalling in vivo, the death of trigeminal ganglion neurones occurs independently of BAX when they are deprived of NGF/TrkA signalling. We conclude that BAX is not universally required for neuronal death induced by neurotrophin deprivation, but that there are major differences for the requirement for BAX among different populations of NGF-dependent neurones.

Key words: BAX, NGF, TrkA, Apoptosis, Sensory neurone, Sympathetic neurone, Mouse

### INTRODUCTION

In the developing vertebrate peripheral nervous system, neurones are generated in excess and those that are superfluous to requirement are lost during a phase of programmed cell death that occurs shortly after they innervate their targets (Oppenheim, 1991). Nerve growth factor (NGF) is the founder member of a family of structurally related secreted proteins termed neurotrophins that promote and regulate the survival of many kinds of neurones in the peripheral nervous system during this stage of development. NGF is required for the survival of sympathetic neurones and a subset of nociceptive neurones, because these neurones are eliminated in developing rodents treated with function-blocking anti-NGF antibodies (Levi-Montalcini, 1987), and are lost in mice that are homozygous for null mutations in the *Ngf* gene (Crowley et al., 1994) or the *TrkA* gene (*Ntrk1* – Mouse Genome Informatics), which encodes the NGF receptor tyrosine kinase (Smeyne et al., 1994). NGF is synthesised in the peripheral target tissues of NGF-dependent neurones in proportion to their innervation density (Korsching and Thoenen, 1983; Harper and Davies, 1990) and administration of excess NGF prevents naturally occurring

cell death within populations of NGF-dependent neurones during development (Levi-Montalcini, 1987).

Insufficient NGF triggers a series of molecular interactions within NGF-dependent neurones that culminate in activation of members of the caspase family of proteases, which dismantle the cell during apoptosis (Jacobson et al., 1997; Thornberry and Lazebnik, 1998). Proteins of the Bcl2 family play a key role in controlling the activation of caspases (Korsmeyer, 1999; Vaux and Korsmeyer, 1999). These proteins fall into two groups that generally either repress apoptosis (Bcl2, Bcl-x<sub>L</sub>, Bcl-w, A1 and Mcl1) or promote apoptosis (BAX, Bcl-x<sub>s</sub>, BAK, BAD, BID, BIK, BOK, BIM and BLK). They influence caspase activation in part by controlling the release of cytochrome c from mitochondria, which interacts with the adapter protein Apaf-1 that, in turn, activates pro-caspase 9 (Li et al., 1997; Qin et al., 1999). For example, BAX increases mitochondrial permeability allowing cytochrome c to pass into the cytosol, whereas Bcl2 and Bcl-x<sub>L</sub> (Bcl2l – Mouse Genome Informatics) prevent cytochrome c release (Kharbanda et al., 1997; Kluck et al., 1997; Yang et al., 1997; Shimizu et al., 1999). In addition, Bcl-x<sub>L</sub> also promotes cell survival by binding directly to Apaf1, preventing it from activating pro-caspase 9 (Hu et al., 1998; Pan et al., 1998), and

Bcl2 also regulates the activation of membrane-associated procaspase 3, independently of cytochrome c (Krebs et al., 1999).

BAX has been shown to play a key role in bringing about neuronal apoptosis in the developing mammalian nervous system. It has been reported that naturally occurring cell death is virtually eliminated in vivo in peripheral ganglia and a wide variety of sites in the brain of BAX-deficient embryos (Deckwerth et al., 1996; White et al., 1998; Patel et al., 2000). Reduction or elimination of BAX expression promotes the in vitro survival of sympathetic neurones and motoneurones in the absence of neurotrophic factors (Deckwerth et al., 1996; Gillardon et al., 1996; Bar-Peled et al., 1999). Overexpression of BAX in cultured sympathetic neurones induces apoptosis in the presence of NGF and increases the rate of apoptosis after NGF withdrawal (Vekrellis et al., 1997; Martinou et al., 1998). These findings have given rise to the view that the expression of BAX is a universal requirement for neuronal death induced by neurotrophic factor deprivation.

To investigate further the physiological validity of this view, we have studied neuronal death in vivo in two populations of NGF-dependent neurones at several stages of development in mice that lack both BAX and NGF or BAX and TrkA. The trigeminal ganglion is a large population of cutaneous sensory neurones whose neuronal complement peaks at E14 and decreases by about 40% by birth as a consequence of naturally occurring neuronal death (Davies and Lumsden, 1984). The earliest trigeminal neurones start responding to NGF when their axons reach their peripheral targets at E11, and from E12 onwards the great majority of neurones depend for their survival on NGF and TrkA signalling both in vivo and in vitro (Davies et al., 1987; Buchman and Davies, 1993; Piñón et al., 1996). The superior cervical ganglion (SCG) is a large population of sympathetic neurones whose neuronal complement peaks at E18 and decreases thereafter as a consequence of naturally occurring neuronal death (Levi-Montalcini, 1987). The earliest SCG neurones start responding to NGF at or shortly after E14 (Coughlin and Collins, 1985; Wyatt and Davies, 1995), and during the period of naturally occurring neuronal death the survival of these neurones is dependent in vivo on both NGF and neurotrophin 3 (NT3) (Levi-Montalcini, 1987; Crowley et al., 1994; Zhou and Rush, 1995; Wyatt et al., 1997; Francis et al., 1999). These neurotrophins promote the survival of SCG neurones by acting via the same receptor TrkA (Davies et al., 1995; Fagan et al., 1996; Belliveau et al., 1997; Wyatt et al., 1997), and absence of either neurotrophin during the period of naturally occurring neuronal death results in the elimination of the majority of the neurones (Levi-Montalcini, 1987; Crowley et al., 1994; Ernfors et al., 1994; Farinas et al., 1994; Zhou and Rush, 1995).

Our studies have revealed that whereas BAX-deficient SCG neurones fail to die in the absence of NGF and TrkA signalling, the enhanced death of trigeminal neurones that occurs in the absence of NGF and TrkA signalling still takes place if BAX is also eliminated. These results indicate that BAX is not universally required for neuronal death induced by neurotrophin deprivation, but that there are major differences for the requirement for BAX among different populations of NGF-dependent neurones.

## MATERIALS AND METHODS

### Experimental animals

Mice that were heterozygous for a null mutation in the *Bax* gene (Knudson et al., 1995) were crossed with mice that were heterozygous for null mutations in the *Ngf* gene (Knudson et al., 1995) to obtain mice that were heterozygous for both null alleles. These double heterozygous mice were bred to obtain litters that contained *Bax*<sup>+/+</sup> *Ngf*<sup>+/+</sup>, *Bax*<sup>-/-</sup> *Ngf*<sup>+/+</sup>, *Bax*<sup>+/+</sup> *Ngf*<sup>-/-</sup> and *Bax*<sup>-/-</sup> *Ngf*<sup>-/-</sup> embryos. *Bax*<sup>+/+</sup> mice were also crossed with mice that were heterozygous for null mutations in the *TrkA* gene (Smeyne et al., 1994) to obtain mice that were heterozygous for both null alleles. These double heterozygous mice were bred to obtain litters that contained *Bax*<sup>+/+</sup> *TrkA*<sup>+/+</sup>, *Bax*<sup>-/-</sup> *TrkA*<sup>+/+</sup>, *Bax*<sup>+/+</sup> *TrkA*<sup>-/-</sup> and *Bax*<sup>-/-</sup> *TrkA*<sup>-/-</sup> embryos. Although embryos of other genotypes resulted from these crosses, these were not used for further analysis. 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). P1 neonates were killed by CO<sub>2</sub> intoxication. The genotypes of the embryos and neonates were determined by a PCR-based technique using DNA extracted from dissected tissues. All mice had been extensively back-crossed into a CD1 background before the generation and crossing of the double heterozygous mice used in this study.

### Quantification of the neuronal complement of SCG and trigeminal ganglia

Neurones were positively identified in histological sections by  $\beta$ -tubulin class III immunohistochemistry (Easter et al., 1993; Moody et al., 1993; Lee and Pixley, 1994; Memberg and Hall, 1995; Moskowitz and Oblinger, 1995). The base of the skull with the contained SCG and trigeminal ganglia was fixed for up to 1 hour at 4°C in Carnoy's fluid (60% ethanol, 30% chloroform, 10% acetic acid), dehydrated through a graded alcohol series, cleared in chloroform and paraffin wax embedded. Serial sections were cut at 8  $\mu$ m and mounted on poly-lysine-coated Gold plus slides (BDH). The sections were cleared in xylene, rehydrated and washed in PBS. Endogenous peroxidase activity was quenched using 1% hydrogen peroxide in methanol. The sections were rinsed in PBS followed by incubation with 10% horse serum plus 0.5% Triton X-100 in PBS to reduce nonspecific binding, and incubated in a humidified chamber for 1 hour at room temperature with anti-neurone-specific  $\beta$ -tubulin class 3 antibody (Promega) diluted in 1:10,000 in PBS. The sections were rinsed with PBS and incubated with biotinylated horse anti-mouse antiserum diluted 1:200 in PBS for 1 hour at room temperature followed by further rinses with PBS and incubation for 30 minutes at room temperature with an avidin/biotinylated horseradish peroxidase macromolecular complex (Vectastain ABC kit; Vector Laboratories). The substrate used for the peroxidase reaction was 1 mg/ml diaminobenzidine tetrachloride (DAB, Sigma) in TBS. The DAB reaction was enhanced using 0.5% copper sulphate in 0.85% sodium chloride. Neurones were recognised by dark-brown cytoplasmic staining. Estimates of neurone number were carried out using a stereology system that uses a combination of the optical disector and volume fraction/Cavaleiri methods (Kinetics Imaging).

### Quantification of neuronal death in the SCG and trigeminal ganglia

To evaluate the extent of cell death two approaches were taken. First, in the histological sections used for counting total neurones (above), all pyknotic nuclei were routinely counted at 400 $\times$  magnification in every fifth section along the entire rostrocaudal extent of the SCG and trigeminal ganglia. Estimates of the total number of pyknotic nuclei in each ganglion were obtained by multiplying the sum of these counts by five.

### Neurone cultures

To compare the survival of Bax-deficient trigeminal and SCG neurones in culture, *Bax*<sup>+/-</sup> mice were crossed and separate dissociated cultures of E16 trigeminal and E18 SCG neurones were set up from individual embryos in the resulting litters (Davies et al., 1995). The genotype of these embryos was subsequently determined by PCR. Dissected ganglia were trypsinised (0.1% trypsin for 15 minutes at 37°C) and dissociated by trituration. The neurones were grown in defined, serum-free medium on a poly-ornithine/laminin substratum in 35 mm diameter tissue culture petri dishes (Davies et al., 1993). Six dishes of trigeminal neurones and six dishes of SCG neurones were set up from each embryo. Purified recombinant NGF

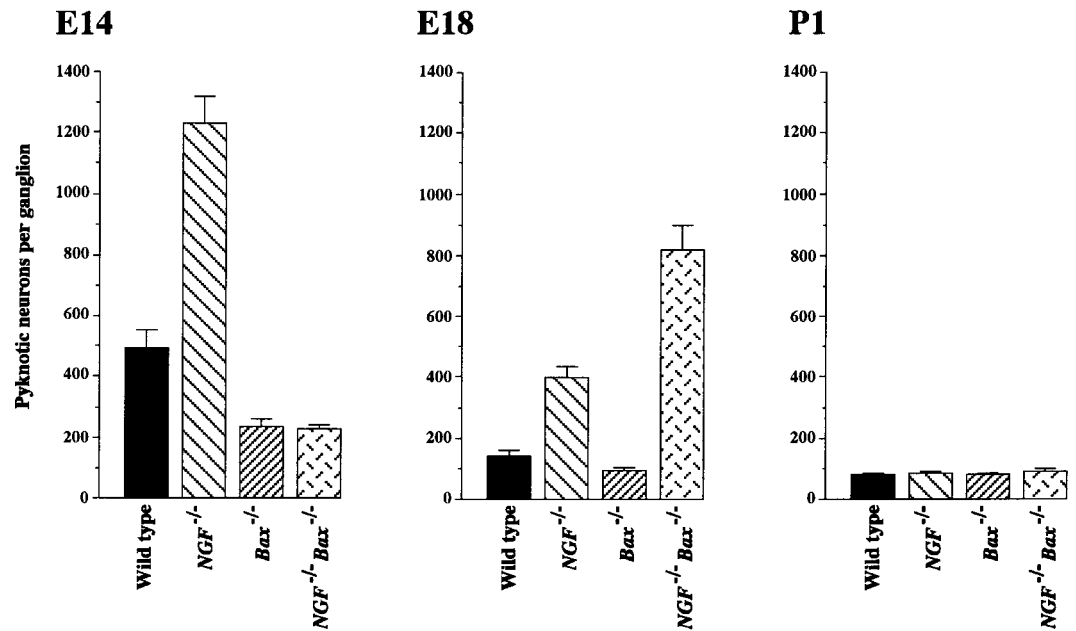
(10 ng/ml) was added to half of these dishes at the time of plating, whereas the other half received no neurotrophic factors (control cultures). The cultures were incubated at 37°C in a 5% CO<sub>2</sub> incubator.

The number of attached neurones within a 12×12 mm square in the centre of each dish was counted 3 hours after plating. The mean of these counts was taken as the initial number of neurones in the experiment. The number of surviving neurones was counted at daily intervals for up to 5 days in the same 12×12 mm area in each dish and is expressed as a percentage of the number of neurones counted at 3 hours.

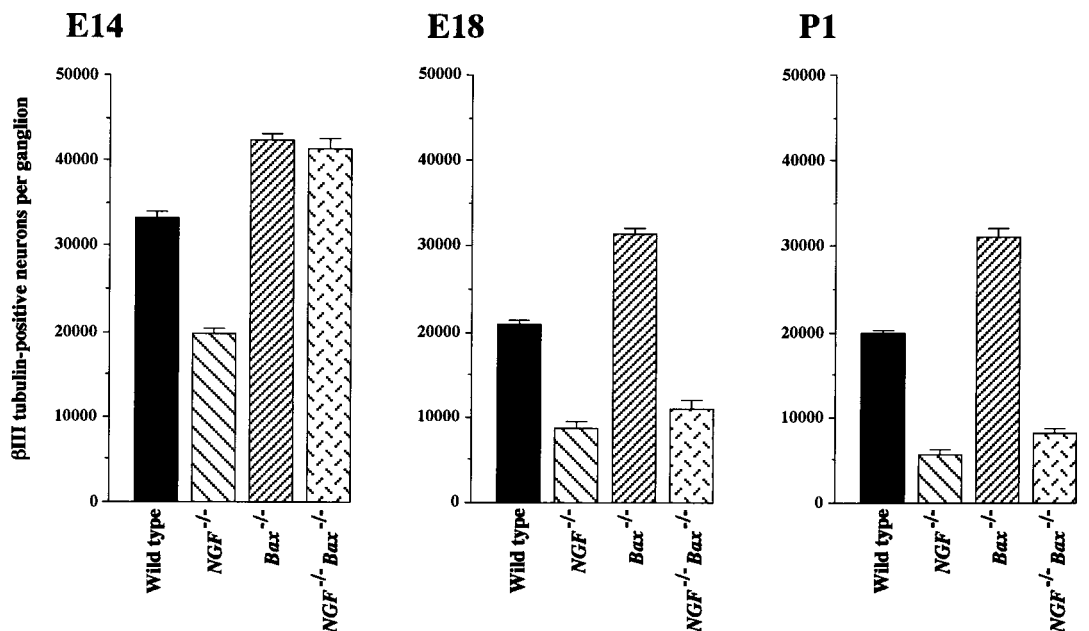
### Immunohistochemical localisation of Bax

For Bax staining, the tissue was fixed, embedded and sectioned as

### Pyknotic neurons in the trigeminal ganglion

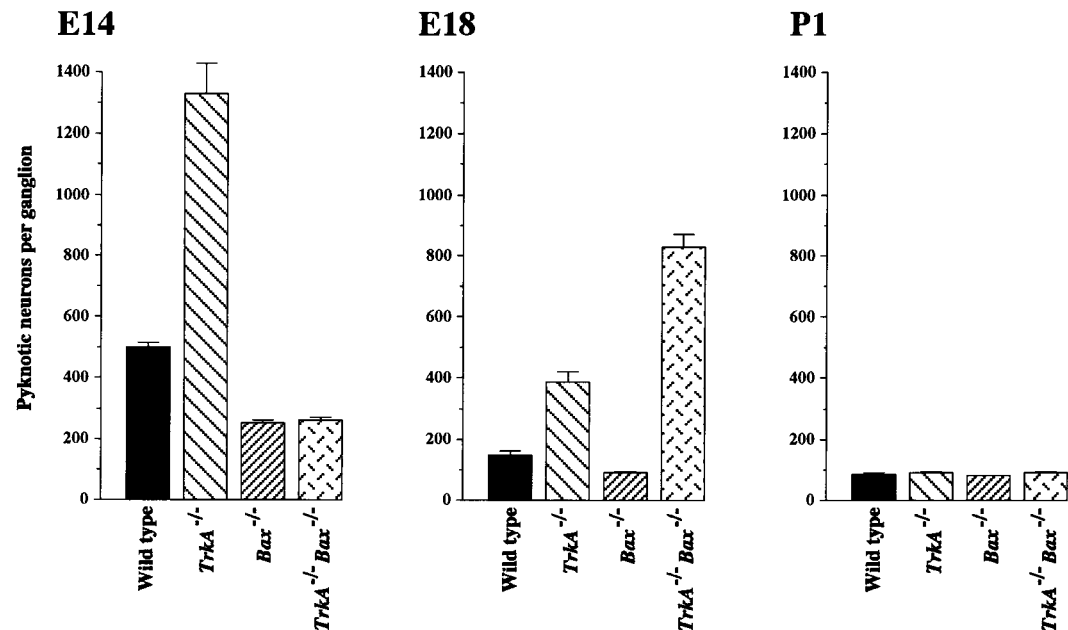


### Total neurons in the trigeminal ganglion

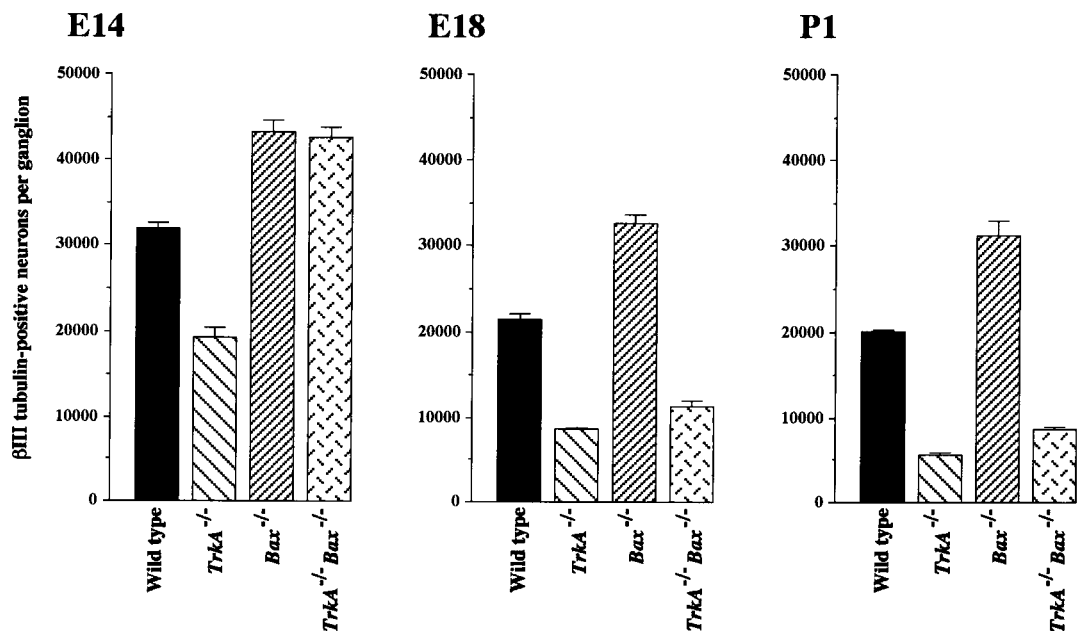


**Fig. 1.** The numbers of neurones with pyknotic nuclei and the total number of neurones in the SCG of wild-type, *Ngf*<sup>-/-</sup>, *Bax*<sup>-/-</sup> and *Ngf*<sup>-/-</sup> *Bax*<sup>-/-</sup> mice at E14, E18 and P1 in litters resulting from crossing *Ngf*<sup>+/-</sup> *Bax*<sup>+/-</sup> mice. The mean and s.e.m. of counts from both SCG of three to five embryos of each genotype at each age are shown.

Pyknotic neurons in the trigeminal ganglion



Total neurons in the trigeminal ganglion



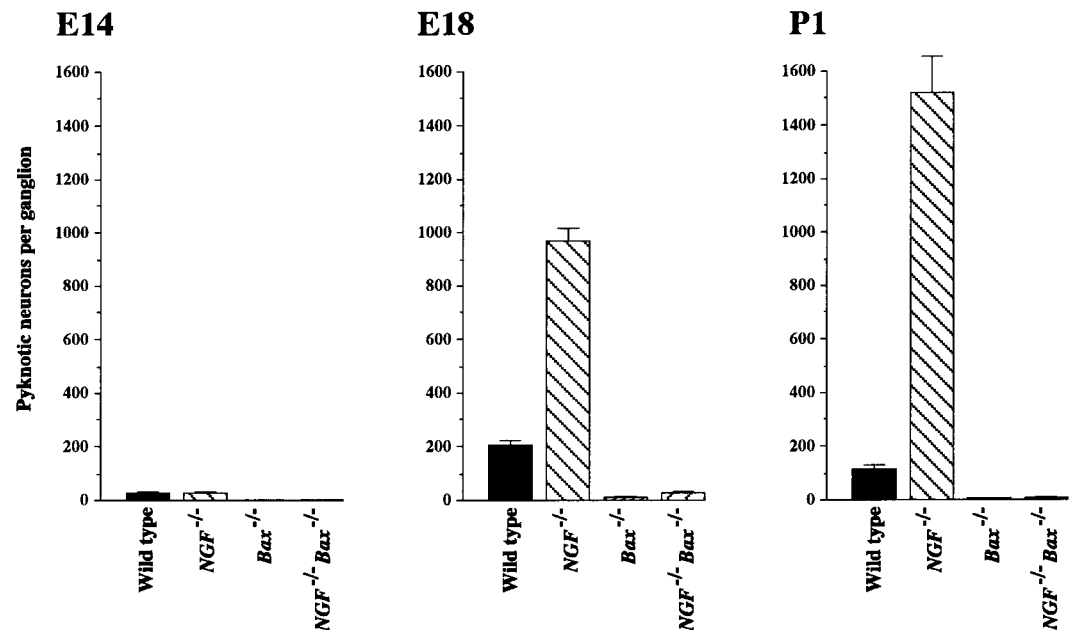
**Fig. 2.** The numbers of neurones with pyknotic nuclei and the total number of neurones in the SCG of wild-type, *TrkA*<sup>-/-</sup>, *Bax*<sup>-/-</sup> and *TrkA*<sup>-/-</sup> *Bax*<sup>-/-</sup> mice at E14, E18 and P1 in litters resulting from crossing *TrkA*<sup>+/-</sup> *Bax*<sup>+/-</sup> mice. The means and s.e.m. of counts from both SCG of three to five embryos of each genotype at each age are shown.

described above. After rehydration, the sections were quenched (3% hydrogen peroxide, 10% methanol in PBS) for 20 minutes. Nonspecific interactions were blocked using 10% horse serum in PBS with 0.04% Triton X-100 at room temperature for 1 hour. The sections were incubated with mouse anti-Bax (Zymed) for 2 hours at 37°C (1:250 in PBS). Nonspecific interactions were blocked using 10% horse serum in PBS with 0.4% Triton X-100 at room temperature for 3 hours. After washing, the cells were labelled with horse anti-mouse biotinylated secondary antibody (1:200) and avidin/biotinylated horse radish peroxidase macromolecular complex (Vectastain ABC Elite Kit, Vector Laboratories). The substrate used for the peroxidase reaction was NovaRed (Vector Laboratories). After staining, the sections were washed in tap water before rehydration and mounting.

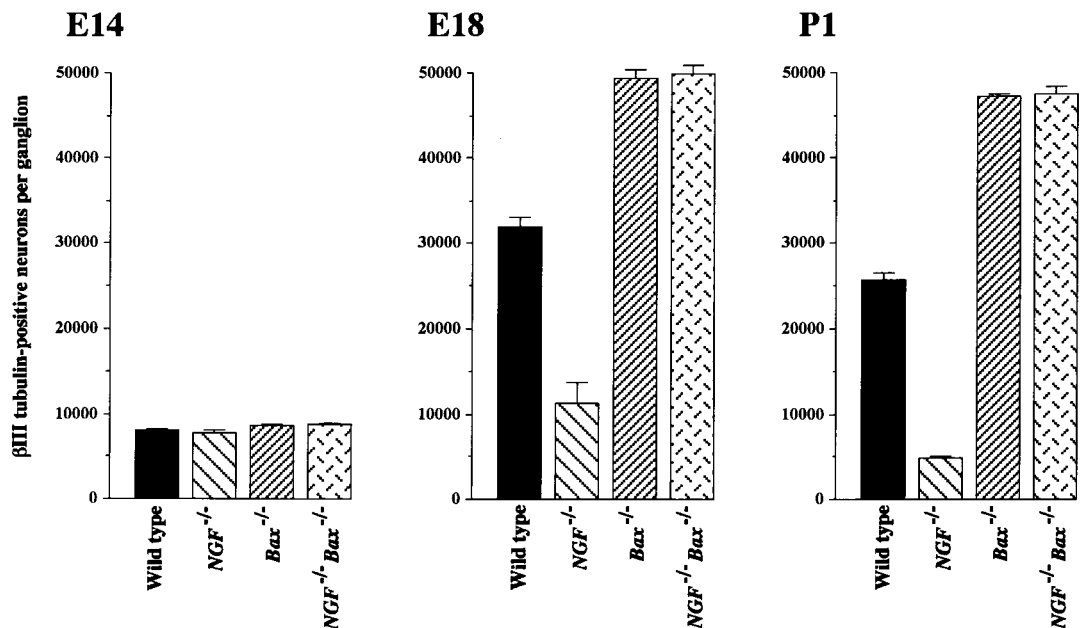
RESULTS

*Bax*<sup>+/-</sup> *Ngf*<sup>+/-</sup> mice were crossed to obtain wild-type mice and mice that possessed different combinations of *Bax* and *Ngf* null alleles, and *Bax*<sup>+/-</sup> *TrkA*<sup>+/-</sup> mice were crossed to obtain wild-type mice and mice that possessed different combinations of *Bax* and *TrkA* null alleles. E14 and E18 embryos and P1 neonates resulting from these crosses were fixed and processed for histology. Tissue from these animals was taken for genotyping to identify the wild-type animals and animals that were homozygous for each null allele on its own or were doubly homozygous for *Bax* and *Ngf* or *Bax* and *TrkA* null

### Pyknotic neurons in the SCG



### Total neurons in the SCG



**Fig. 3.** The numbers of neurones with pyknotic nuclei and the total number of neurones in the trigeminal ganglia of wild-type, *Ngf*<sup>-/-</sup>, *Bax*<sup>-/-</sup> and *Ngf*<sup>-/-</sup> *Bax*<sup>-/-</sup> mice at E14, E18 and P1 in litters resulting from crossing *Ngf*<sup>+/-</sup> *Bax*<sup>+/-</sup> mice. The means and s.e.m. of counts from both trigeminal ganglia of three to five embryos of each genotype at each age are shown.

alleles, as only animals with these genotypes were serially sectioned to count the number of neurones (positively identified by staining with anti- $\beta$ -tubulin class III antibodies) and neurones with pyknotic nuclei in the SCG and trigeminal ganglia. To avoid observer bias, all histology slides were coded, and the codes were only broken when the counts had been completed.

#### Neuronal survival and death

##### SCG

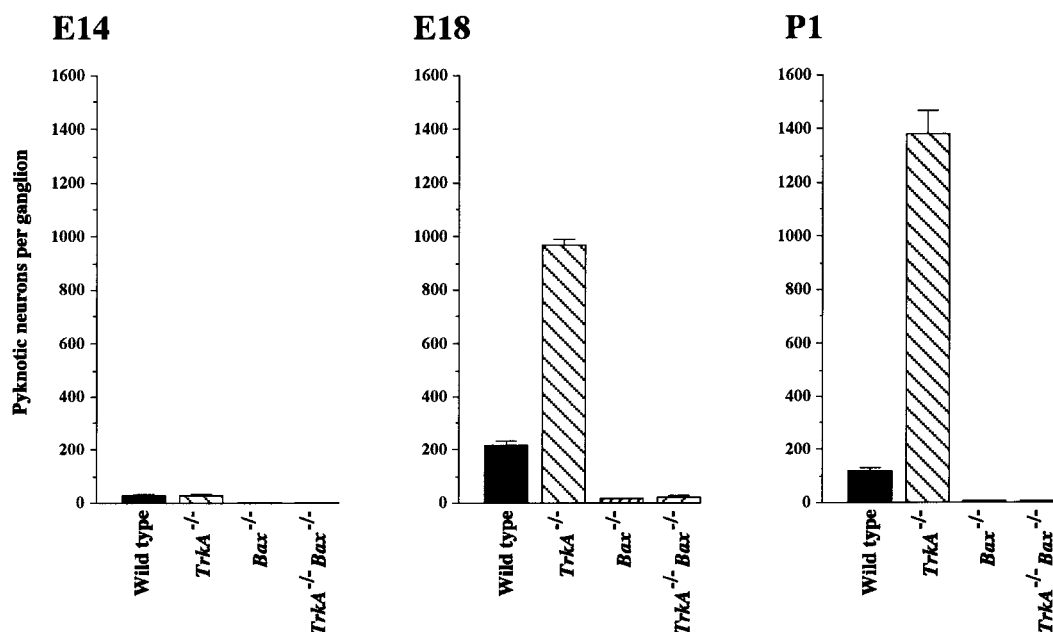
Counts of the numbers of neurones in the SCG of wild-type

embryos resulting from both sets of crosses revealed a fourfold increase from E14 to E18, consistent with the generation of many neurones from proliferating neuroblasts during and shortly after E14 (Maina et al., 1998). There was a decrease in the neuronal complement of the SCG between E18 and P1 that was associated with an elevated number of dying neurones in the ganglion, indicating that naturally occurring neuronal death is taking place in the ganglion during this period of development.

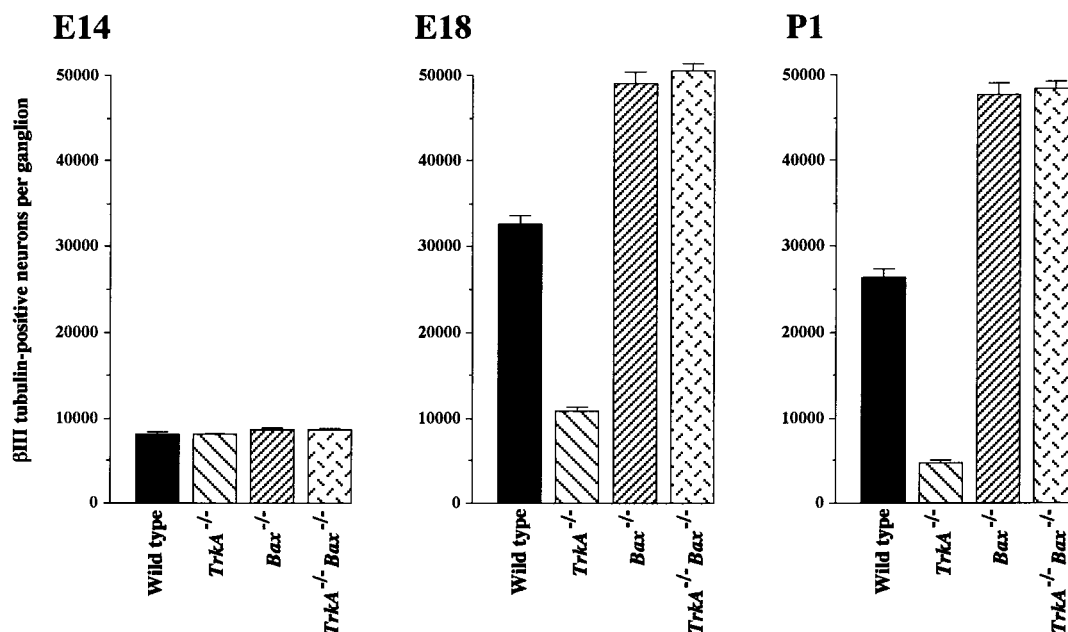
Animals lacking NGF or TrkA displayed a marked increase in the number of neurones undergoing cell death in the SCG



### Pyknotic neurons in the SCG



### Total neurons in the SCG

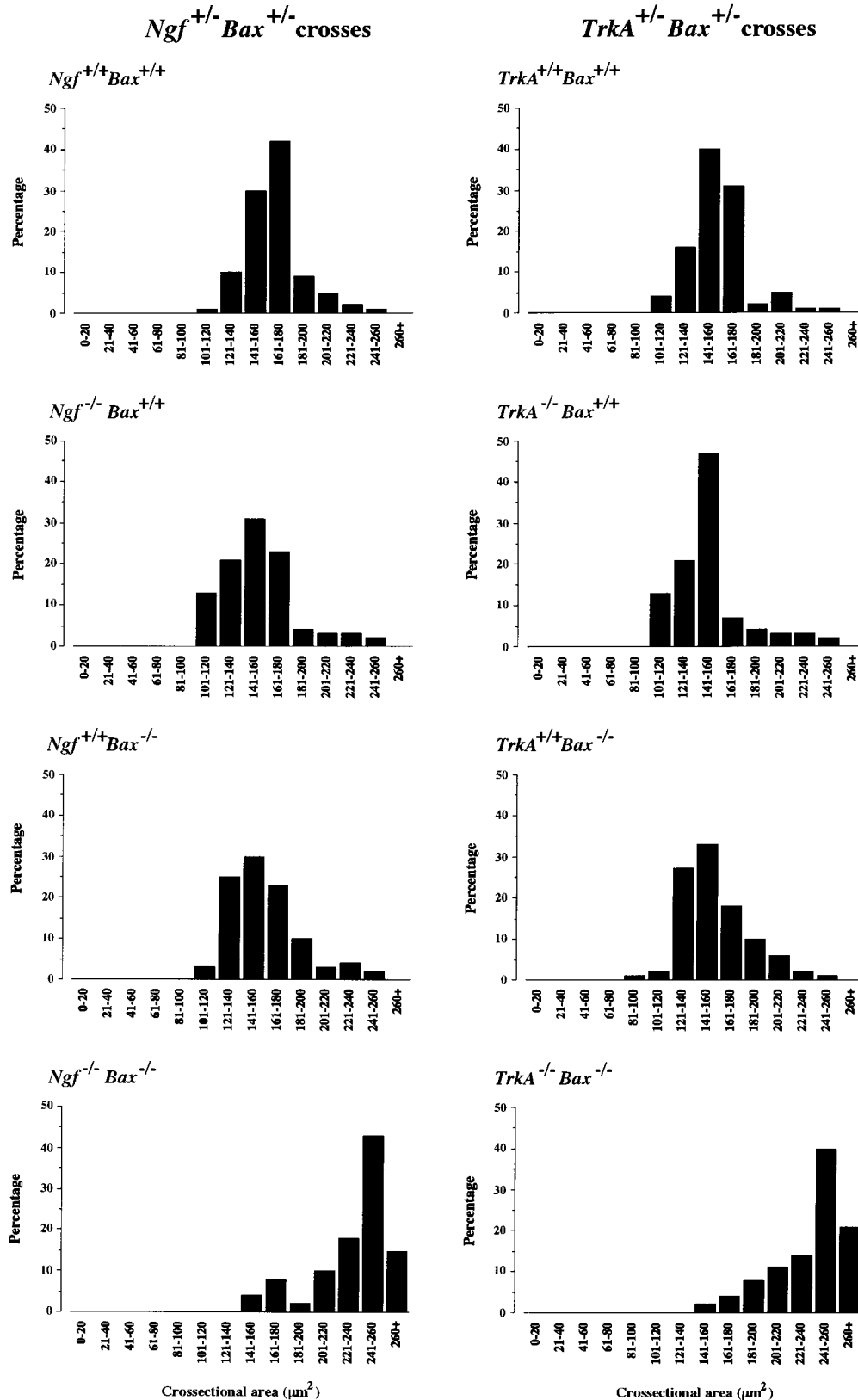


**Fig. 4.** The numbers of neurones with pyknotic nuclei and the total number of neurones in the SCG of wild-type, *TrkA*<sup>-/-</sup>, *Bax*<sup>-/-</sup> and *TrkA*<sup>-/-</sup> *Bax*<sup>-/-</sup> mice at E14, E18 and P1 in litters resulting from crossing *TrkA*<sup>+/-</sup> *Bax*<sup>+/-</sup> mice. The means and s.e.m. of counts from both trigeminal ganglia of three to five embryos of each genotype at each age are shown.

and a corresponding decrease in the neuronal complement of the ganglion that was virtually identical in both genotypes (Fig. 1, Fig. 2). Although no significant differences were evident between *Ngf*<sup>-/-</sup>, *TrkA*<sup>-/-</sup> and wild-type embryos at E14, by E18 the numbers of neurones with pyknotic nuclei were almost fivefold higher in the SCG of *Ngf*<sup>-/-</sup> and *TrkA*<sup>-/-</sup> embryos than in wild-type embryos and the numbers of neurones remaining in the ganglia were threefold lower than in wild-type ganglia. There was a further increase in the number of dying neurones in the SCG of *Ngf*<sup>-/-</sup> and *TrkA*<sup>-/-</sup> mice between E18 and P1, reaching a level that was 12-fold higher than in wild-type

neonates. At P1, the number of neurones remaining in the SCG of *Ngf*<sup>-/-</sup> and *TrkA*<sup>-/-</sup> mice was more than fivefold lower than in wild-type neonates. These results confirm that during the period of naturally occurring neuronal death in the SCG, the survival of the majority of neurones is dependent on a supply of NGF acting via the TrkA receptor.

Neuronal death was virtually eliminated in the SCG of embryos and neonates that were homozygous for a null mutation in the *Bax* gene (Fig. 1, Fig. 2). Hardly any pyknotic cells were observed in the SCG of *Bax*<sup>-/-</sup> mice at any of the ages examined. Although the number of neurones in the SCG



**Fig. 5.** The cross sectional areas of the cell bodies of P1 SCG neurones (at the level of the nucleolus) of wild-type, *Ngf*<sup>-/-</sup>, *Bax*<sup>-/-</sup> and *Ngf*<sup>-/-</sup> *Bax*<sup>-/-</sup> mice resulting from crossing *Ngf*<sup>+/+</sup> *Bax*<sup>+/+</sup> mice and wild-type, *TrkA*<sup>-/-</sup>, *Bax*<sup>-/-</sup> and *TrkA*<sup>-/-</sup> *Bax*<sup>-/-</sup> mice resulting from crossing *TrkA*<sup>+/+</sup> *Bax*<sup>+/+</sup> mice. The percentage of neurones whose cross sectional area lies within defined limits are shown. The data are compiled from measurements derived from at least 100 neurones taken at random in the SCG of three neonates of each genotype.

P1. Furthermore, there was no significant decrease in the number of neurones in the SCG of *Bax*<sup>-/-</sup> embryos between E18 and P1 ( $P > 0.1$ ,  $t$ -tests). These results demonstrate that BAX expression is required for the death of SCG neurones during the phase of naturally occurring neuronal death.

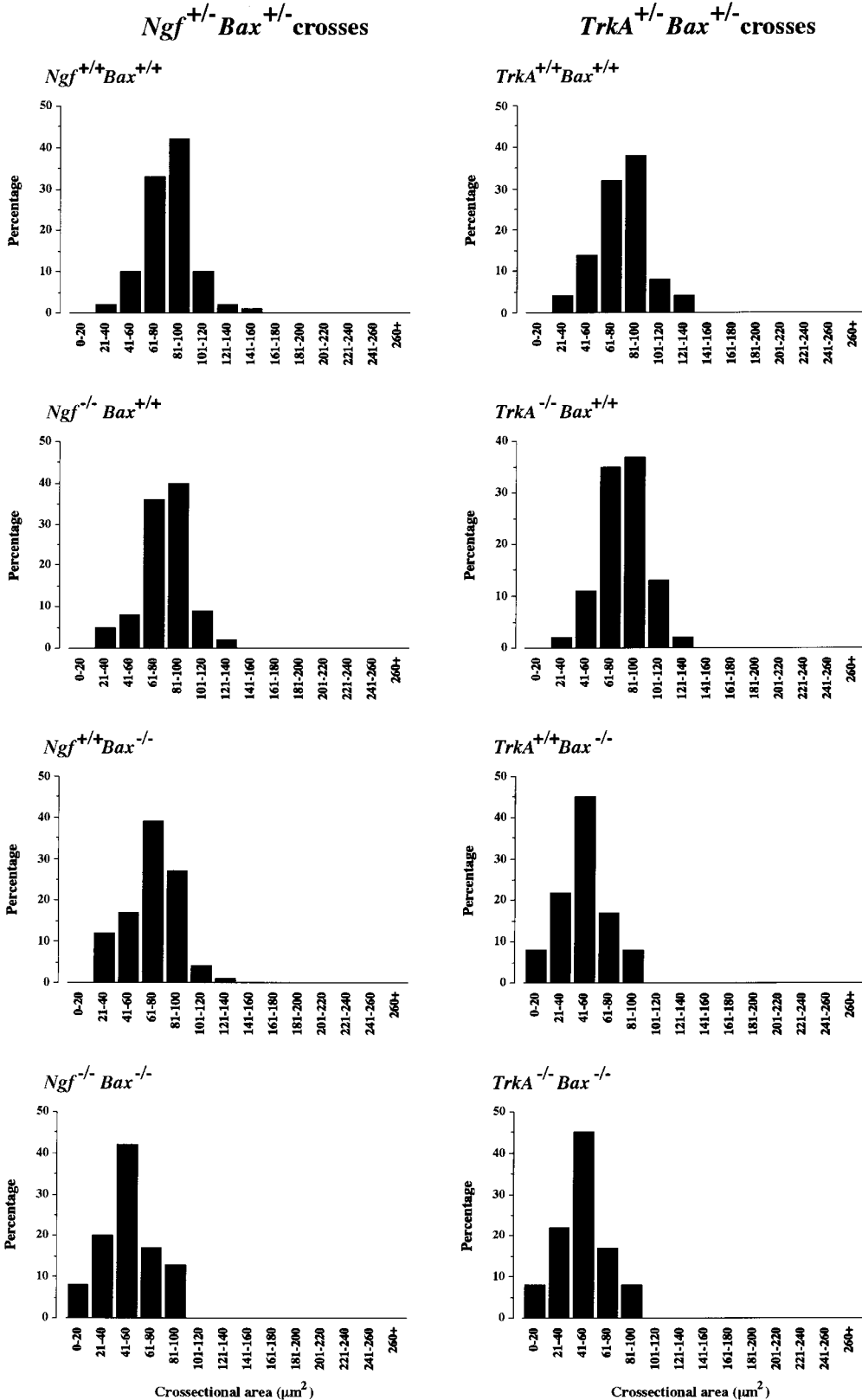
The enhanced death of neurones that occurred in the SCG of mice lacking NGF or TrkA was completely prevented by the concomitant elimination of BAX (Fig. 1, Fig. 2). Hardly any pyknotic cells were observed in the SCG of *Bax*<sup>-/-</sup> *Ngf*<sup>-/-</sup> and *Bax*<sup>-/-</sup> *TrkA*<sup>-/-</sup> mice at any of the ages examined. Accordingly, the number of neurones in the SCG of *Bax*<sup>-/-</sup> *Ngf*<sup>-/-</sup> and *Bax*<sup>-/-</sup> *TrkA*<sup>-/-</sup> mice was markedly elevated above the numbers in wild-type mice at E18 and P1, and there were no significant differences between the numbers of SCG neurones in *Bax*<sup>-/-</sup>, *Bax*<sup>-/-</sup> *Ngf*<sup>-/-</sup> and *Bax*<sup>-/-</sup> *TrkA*<sup>-/-</sup> at all ages studied. These results suggest that the enhanced neuronal death that occurs in the SCG of mice lacking either NGF or TrkA is completely dependent on BAX.

#### Trigeminal ganglia

Counts of the numbers of surviving and dying neurones in the trigeminal ganglia of wild-type embryos resulting from both

of *Bax*<sup>-/-</sup> embryos was not significantly different from that in wild-type mice at E14, there were over 50% more neurones in the SCG of E18 *Bax*<sup>-/-</sup> embryos than in wild-type embryos, and this difference increased to over 80% more neurones by

sets of crosses at the three stages of development studied (Fig. 3, Fig. 4) were very similar to previously reported estimates (Davies and Lumsden, 1984; Piñón et al., 1996; Piñón et al., 1997; Middleton et al., 2000). Between E14 and P1, there was



**Fig. 6.** The cross sectional areas of the cell bodies of P1 trigeminal neurones (at the level of the nucleolus) of wild-type, *Ngf*<sup>-/-</sup>, *Bax*<sup>-/-</sup> and *Ngf*<sup>-/-</sup> *Bax*<sup>-/-</sup> mice resulting from crossing *Ngf*<sup>+/+</sup> *Bax*<sup>+/+</sup> mice and wild-type, *TrkA*<sup>-/-</sup>, *Bax*<sup>-/-</sup> and *TrkA*<sup>-/-</sup> *Bax*<sup>-/-</sup> mice resulting from crossing *TrkA*<sup>+/+</sup> *Bax*<sup>+/+</sup> mice. The percentage of neurones whose cross sectional area lies within defined limits are shown. The data are compiled from measurements derived from at least 100 neurones taken at random in the trigeminal ganglia of three neonates of each genotype.

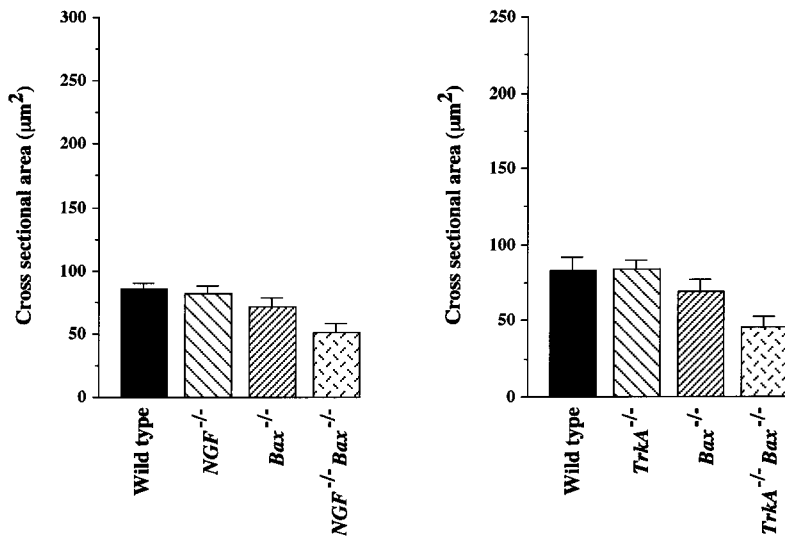
increase in the number of neurones undergoing cell death in the trigeminal ganglia and a corresponding decrease in the neuronal complement of these ganglia that was virtually identical in both genotypes (Fig. 3, Fig. 4). The numbers of neurones with pyknotic nuclei was two- to threefold higher in the trigeminal ganglia of *Ngf*<sup>-/-</sup> and *TrkA*<sup>-/-</sup> embryos at E14 and E18 compared with wild-type ganglia at these ages. This was associated with an approximate 40% decrease in the neuronal complement of the ganglia in *Ngf*<sup>-/-</sup> and *TrkA*<sup>-/-</sup> embryos compared with wild-type embryos at E14 and an approximate 60% reduction compared with wild-type embryos at E18, and by P1 there were 72% fewer neurones in the ganglia of these mice. The numbers of dying neurones had fallen to a low level by P1, and there were no significant differences in the numbers of neurones with pyknotic nuclei in the ganglia of wild-type, *Ngf*<sup>-/-</sup> and *TrkA*<sup>-/-</sup> animals at this age. These results show that during the period of naturally occurring neuronal death in the trigeminal ganglion, the survival of the majority of neurones is dependent on a supply of NGF acting via the TrkA receptor.

The extent of neuronal death in the trigeminal ganglia of BAX-deficient embryos was reduced but not eliminated during the period of naturally occurring neuronal death (Fig. 3, Fig. 4). The number of neurones with pyknotic nuclei in the trigeminal ganglia of *Bax*<sup>-/-</sup> embryos

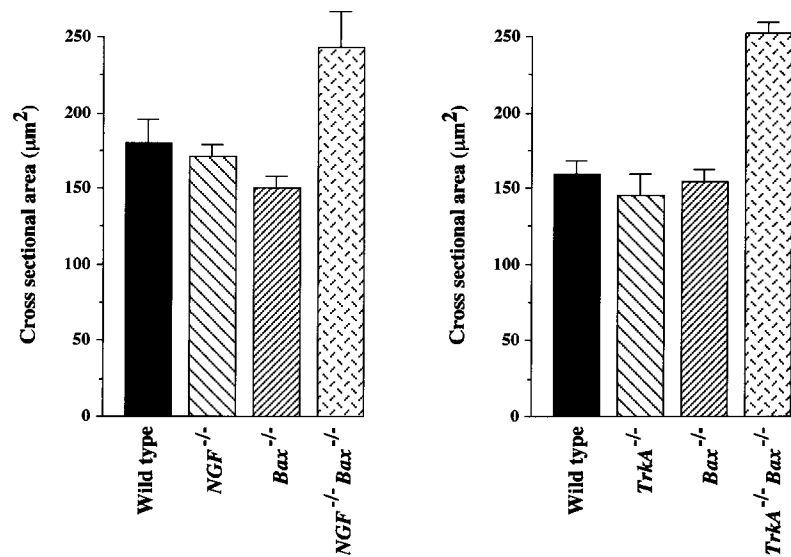
an approximate 40% decrease in the total number of neurones in the ganglion and a marked decrease in the number of neurones with pyknotic nuclei. Animals that lacked NGF or TrkA displayed a marked



## Sympathetic neurons



## Trigeminal neurons



**Fig. 7.** The mean and s.e.m. of measurements of the cross-sectional areas of the cell bodies of P1 SCG and trigeminal neurones (at the level of the nucleolus) of wild-type, *Ngf*<sup>-/-</sup>, *Bax*<sup>-/-</sup> and *Ngf*<sup>-/-</sup> *Bax*<sup>-/-</sup> mice resulting from crossing *Ngf*<sup>+/-</sup> *Bax*<sup>+/-</sup> mice and wild type, *TrkA*<sup>-/-</sup>, *Bax*<sup>-/-</sup> and *TrkA*<sup>-/-</sup> *Bax*<sup>-/-</sup> mice resulting from crossing *TrkA*<sup>+/-</sup> *Bax*<sup>+/-</sup> mice. The data are compiled from measurements derived from at least 100 neurones taken at random in the SCG and trigeminal ganglia of three neonates of each genotype.

was 51% lower at E14, and 36% lower at E18, but not significantly different from wild-type mice at P1. There were 32% more neurones in the trigeminal ganglia of E14 *Bax*<sup>-/-</sup> embryos than in wild-type embryos, and this difference increased to 56% more neurones by P1. There was, however, a clear decrease of 24% in the number of neurones in the trigeminal ganglia of *Bax*<sup>-/-</sup> embryos between E14 and E18, and a further smaller decrease to P1. These results demonstrate that BAX expression is required for the death of a proportion

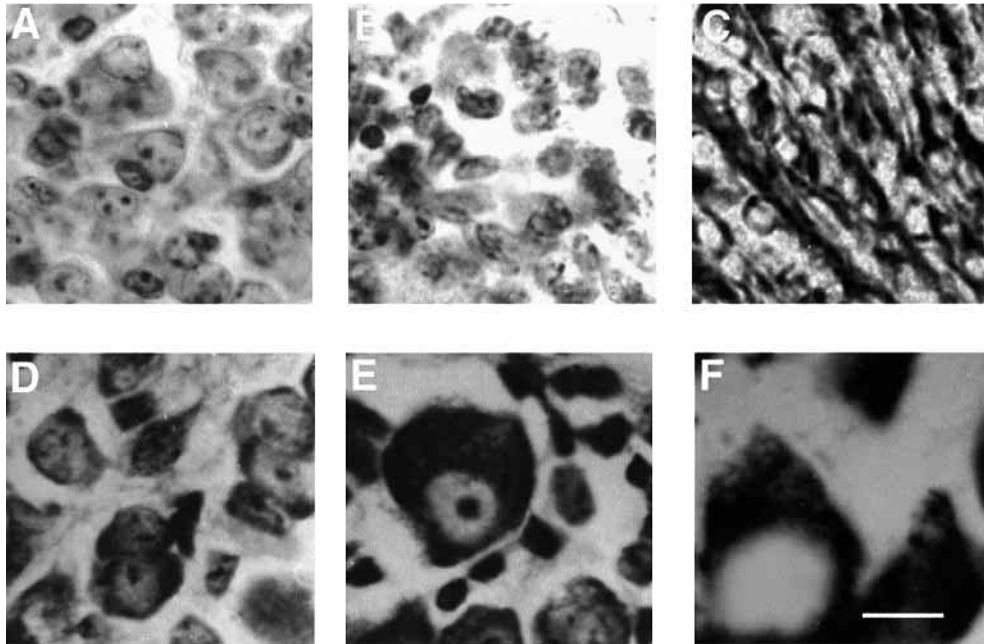
but by no means all neurones in the trigeminal ganglion during the phase of naturally occurring neuronal death.

At E14, the number of surviving and dying neurones in the trigeminal ganglion was not significantly different between embryos lacking BAX alone and embryos lacking BAX and NGF or BAX and TrkA (Fig. 3, Fig. 4). This indicates that at this stage of development, the enhanced loss of neurones that occurs in embryos lacking NGF and TrkA signalling is prevented by the absence of BAX. However, by E18 the number of pyknotic neurones in the trigeminal ganglia of *Bax*<sup>-/-</sup> *Ngf*<sup>-/-</sup> and *Bax*<sup>-/-</sup> *TrkA*<sup>-/-</sup> embryos was almost sixfold higher than in the ganglia of wild-type embryos, and over twofold higher than in the ganglia of embryos lacking either NGF or TrkA alone. Between E14 and E18 there was an almost fourfold decrease in number of neurones in the trigeminal ganglia of *Bax*<sup>-/-</sup> *Ngf*<sup>-/-</sup> and *Bax*<sup>-/-</sup> *TrkA*<sup>-/-</sup> embryos, so that by E18 there were similar numbers of neurones in the ganglia of these embryos and embryos that lacked either NGF or TrkA alone. This indicates that the survival of trigeminal neurones deprived of NGF or TrkA signalling is not sustained by the absence of BAX. There was a further fall in the number of neurones in the trigeminal ganglia of *Bax*<sup>-/-</sup> *Ngf*<sup>-/-</sup> and *Bax*<sup>-/-</sup> *TrkA*<sup>-/-</sup> mice between E18 and P1. By P1, the number of dying neurones in the trigeminal ganglia of *Bax*<sup>-/-</sup> *Ngf*<sup>-/-</sup> and *Bax*<sup>-/-</sup> *TrkA*<sup>-/-</sup> mice was not significantly different from *Ngf*<sup>-/-</sup>, *TrkA*<sup>-/-</sup> and wild-type mice. Taken together, these findings indicate that the excessive neuronal death that occurs in the trigeminal of mice that lack either NGF or TrkA signalling is initially delayed by the absence of BAX, but subsequently occurs to virtually the same extent, independently of BAX.

Because these results were so unexpected, we wanted to be sure that all neurones were being positively identified in histological sections. In addition to using antibodies to  $\beta$ -tubulin class III to identify neurones, we also used several other neuronal markers. In sections stained with anti-neurofilament antibodies (to neurofilament H, neurofilament M and neurofilament L, either separately or in combination) or anti-peripherin antibodies, no more neurones were identified in sections of trigeminal ganglia than with anti- $\beta$ -tubulin class III antibodies. Indeed, on sections of SCG and trigeminal ganglia of all genotypes and at all three ages studied,  $\beta$ -tubulin staining was

consistently more effective in identifying neurones than either neurofilament or peripherin staining.

Furthermore, because our demonstration that BAX is not required for the death of trigeminal sensory neurones induced by absence of NGF/TrkA signalling *in vivo* contrasts with the recent report that absence of BAX results in the survival of dorsal root ganglion (DRG) sensory neurones that would otherwise die in the absence of NGF/TrkA signalling (Patel et al., 2000), we also studied neuronal death in the DRG of our



**Fig. 8.** Photomicrographs illustrating the typical appearance of P1 neurones in sections of the wild-type SCG (A), *Ngf*<sup>-/-</sup> *Bax*<sup>-/-</sup> SCG (B), wild-type trigeminal ganglia (D) and *Ngf*<sup>-/-</sup> *Bax*<sup>-/-</sup> trigeminal ganglia (E). The appearance of P1 *Ngf*<sup>-/-</sup> *Bax*<sup>-/-</sup> SCG (C) and trigeminal (F) neurones stained for  $\beta$ III tubulin is also shown. Because of the small size of the neurones in C, there are many neurones out of the plane of focus. Those within the plane of focus show the typical cytoplasmic staining for  $\beta$ III tubulin surrounding an unstained nuclear halo. Scale bar: 20  $\mu$ m.

mice. In agreement, the findings of Patel and colleagues in lumbar DRG, we found in cervical DRG that elimination of BAX virtually abolished neuronal death in embryos and neonates lacking either NGF or TrkA (data not shown). These results show that BAX is also required for sensory neurone death induced by absence of NGF/TrkA signalling in the most rostral set of DRG.

### Neuronal morphology

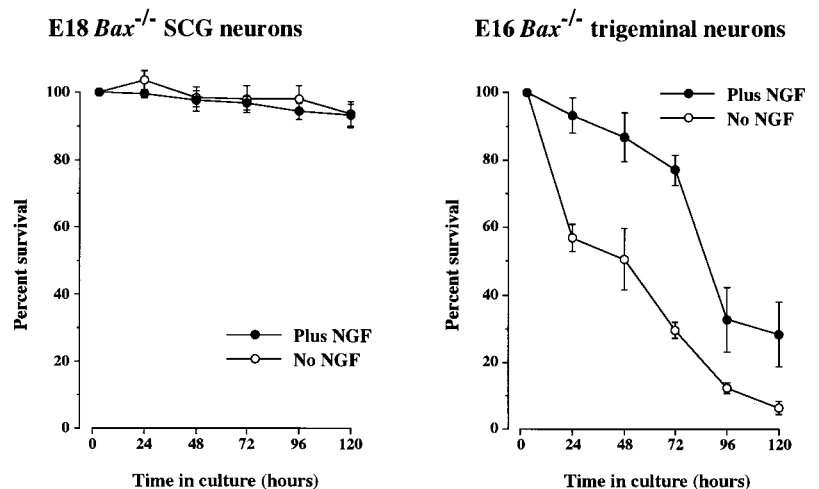
In the course of quantifying neuronal survival and death in the SCG and trigeminal ganglia of BAX-deficient mice in which NGF/TrkA signalling had also been eliminated, we observed and quantified differences in the perikaryon size compared with wild-type mice. To standardise these measurements, the cross-sectional area of the cell body (outlined by  $\beta$ III tubulin immunohistochemistry) was measured at the level of the nucleolus (identified by counterstaining with Haematoxylin).

### SCG

In P1 neonates, the cross-sectional area of the cell bodies of wild-type SCG neurones ranged from 20 to 160  $\mu$ m<sup>2</sup>, with a peak range between 80 and 100  $\mu$ m<sup>2</sup> (Fig. 5). The size of these neurones was very similar in *Ngf*<sup>-/-</sup> and *TrkA*<sup>-/-</sup> neonates, and although the mean cross sectional area of SCG cell bodies in *Bax*<sup>-/-</sup> mice was consistently lower than that of wild-type, *Ngf*<sup>-/-</sup> and *TrkA*<sup>-/-</sup> neonates (Fig. 7) with a peak range between 40 and 80  $\mu$ m<sup>2</sup> (Fig. 5), these differences were not statistically significant ( $P > 0.05$ ,  $t$ -tests). However, the neurones in the SCG of BAX-deficient neonates that additionally lacked either NGF or TrkA were significantly smaller than those of all other genotypes ( $P < 0.001$ ,  $t$ -test). The typical appearance of the SCG neurones in wild type and *Bax*<sup>-/-</sup> *Ngf*<sup>-/-</sup> neonates is illustrated in Fig. 8.

### Trigeminal neurones

In P1 neonates, the cross sectional area of the cell bodies of wild-type trigeminal neurones is considerably greater than that of SCG neurones, and ranged from 100 to 260  $\mu$ m<sup>2</sup>, with a peak range between 140 and 180  $\mu$ m<sup>2</sup> (Fig. 6, Fig. 7). As was the case in the SCG, there were no significant differences in the mean size of neurones in the trigeminal ganglia of wild-type, *Ngf*<sup>-/-</sup>, *TrkA*<sup>-/-</sup> and *Bax*<sup>-/-</sup> ( $P > 0.05$ ,  $t$ -tests). However, in marked contrast to the SCG, the neurones in the trigeminal ganglia of BAX-deficient neonates that additionally lacked either NGF or TrkA were much larger than those of all other genotypes ( $P < 0.01$ ,  $t$ -test). The typical appearance of the trigeminal ganglia neurones in wild-type and *Bax*<sup>-/-</sup> *Ngf*<sup>-/-</sup> neonates is illustrated in Fig. 8.



**Fig. 9.** The percent survival of E18 SCG and E16 trigeminal neurones obtained from *Bax*<sup>-/-</sup> embryos grown for up to 5 days in defined medium without neurotrophic factors or medium containing 2 ng/ml NGF. The means and s.e.m. are shown ( $n = 4$  for each data point).

### Neuronal survival and death in vitro

To investigate further the relative importance of BAX in bringing about apoptosis of SCG and trigeminal neurones deprived of neurotrophic support, we compared the survival of BAX-deficient neurones in low-density dissociated cultures established from these ganglia in defined, serum-free medium with and without NGF. Because there is massive death of trigeminal neurones between E14 and E18 in embryos deficient for both BAX and NGF or BAX and TrkA (Fig. 1, Fig. 2), we established cultures trigeminal neurone cultures from *Bax*<sup>-/-</sup> embryos in the middle of this period at E16. SCG neurones were cultured from *Bax*<sup>-/-</sup> embryos at E18 when substantial numbers of neurones are undergoing apoptosis in embryos that lack either NGF or TrkA (Fig. 3, Fig. 4). It has previously been shown that wild-type E16 trigeminal and E18 SCG neurones cultured under identical conditions all die within 24 hours of plating in the absence of NGF, but are sustained by its presence (Davies et al., 1995).

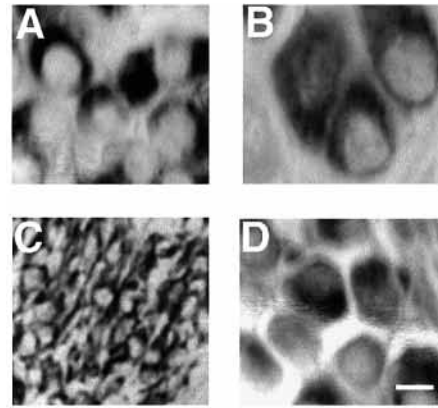
In accordance with the observed survival of BAX-deficient sympathetic neurones in the absence of NGF/TrkA signalling in vivo (Fig. 3, Fig. 4), there was virtually no death of E18 BAX-deficient sympathetic neurones cultured in defined medium without neurotrophic factors for at least 5 days (Fig. 9). In marked contrast, E16 BAX-deficient trigeminal neurones failed to survive in defined medium without neurotrophic factors, so that by 5 days there were less than 10% remaining. Although addition of NGF to the culture medium rescued many of these neurones, more than half of the neurones were dead after 5 days (Fig. 9). These in vitro findings accord with our observation that a substantial proportion of trigeminal neurones die in vivo during this stage in development in embryos that lack BAX together with either NGF or TrkA, and that BAX-deficient trigeminal neurones die at a slower rate in embryos with intact NGF/TrkA signalling (Fig. 1, Fig. 2). The results of a limited number of E14 BAX-deficient trigeminal neurone cultures also revealed that these neurones died without NGF in culture (data not shown).

### BAX is normally expressed in developing sympathetic and trigeminal neurones

To determine if the difference in the importance of BAX in bringing about the death of NGF-deprived neurones in the SCG and trigeminal ganglia is merely due to differences in BAX expression, we used immunohistochemistry to localise BAX protein in sections of these ganglia. Fig. 10 shows that virtually all neurones in both ganglia were immunoreactive for BAX protein at both E14 and P1. No neuronal staining was observed in sections from which the primary antibody was omitted (not shown). This observation indicates that BAX is expressed by both kinds of neurones. Together with the above data, this result implies that there are differences in the function of BAX in these two populations of NGF-dependent neurones.

### DISCUSSION

Our in vivo analysis of BAX-deficient mice that additionally lack either NGF or TrkA has demonstrated that BAX is not a universal requirement for neuronal apoptosis induced by elimination of NGF/TrkA signalling, but that there are major differences in the need for BAX in different populations of



**Fig. 10.** Photomicrographs illustrating the staining for BAX protein in sections of E14 (A) and P1 (B) trigeminal ganglia and E14 (C) and P1 (D) SCG of wild-type mice. Scale bar: 20  $\mu$ m.

NGF-dependent neurones. In agreement with previous studies that have reported that there are increased numbers of neurones and decreased cell death in the SCG of BAX-deficient mice (Deckwerth et al., 1996; White et al., 1998), we have demonstrated that naturally occurring neuronal death is completely eliminated in the SCG of mice lacking BAX. In addition, we have shown that the massive loss of neurones that occurs in this ganglion in the absence of either NGF or TrkA is also completely prevented by the concomitant elimination of BAX. In marked contrast to sympathetic neurones, absence of BAX reduces but by no means eliminates naturally occurring neuronal death in the trigeminal ganglion. More strikingly, the massive loss of trigeminal neurones that occurs in mice lacking either NGF or TrkA, although briefly delayed by the additional absence of BAX, subsequently occurs to the same extent irrespective of the presence of BAX. Accordingly, unlike BAX deficient SCG neurones, which survive without NGF in culture, BAX-deficient neurones die in culture without NGF and are only partially rescued by NGF. These results clearly demonstrate that whereas apoptosis of developing sympathetic neurones deprived of NGF/TrkA signalling is completely dependent on BAX, apoptosis of trigeminal neurones deprived of NGF/TrkA signalling occurs independently of BAX both in vivo and in vitro.

The occurrence of apoptosis by a BAX-independent mechanism in the trigeminal ganglia of NGF/TrkA deficient mice is not typical of other populations of predominantly NGF-dependent sensory neurones. It has recently been shown that elimination of BAX prevents the massive loss of neurones that would otherwise occur in the lumbar DRG of newborn mice lacking either NGF or TrkA (Patel et al., 2000). Likewise, we have found that apoptosis is prevented in the cervical DRG of BAX-deficient mice that lack either NGF or TrkA, making it quite likely that apoptosis of NGF-deprived DRG neurones is dependent on BAX at all axial levels. The occurrence of massive neuronal death by a BAX-independent mechanism in the trigeminal ganglia of mice in which NGF/TrkA signalling has been eliminated suggests that other proteins are involved in bringing about the death of these neurones under conditions of insufficient NGF/TrkA signalling. Moreover, as almost all neurones in the SCG and trigeminal ganglia display similar levels of BAX immunoreactivity, the differences in the



requirement for BAX in bringing about apoptosis in these two populations of neurones does not appear to reflect differences in BAX expression.

BAX causes apoptosis by undergoing a conformational change which results in its oligomerisation and insertion into the outer mitochondrial membrane (Wolter et al., 1997; Gross et al., 1998; Desagher et al., 1999; Eskes et al., 2000). This, in turn, facilitates the release of cytochrome c and other pro-apoptotic proteins from mitochondria, which results in caspase activation and apoptosis (Du et al., 2000; Green, 2000; Verhagen et al., 2000). While the conformational change in BAX that triggers these events can be induced by an interaction between BAX and BID (Desagher et al., 1999), BID can also translocate to mitochondria and induce cytochrome c by a mechanism that does not require BAX (Kim et al., 2001). This BAX-independent effect of BID may be mediated by its interaction with BAK, a Bcl2 family protein that resident in the outer mitochondrial membrane (Griffiths et al., 1999). This interaction causes a conformational change in BAK, which results in its oligomerisation and release of cytochrome c (Wei et al., 2000). In future work it will be important to investigate the potential role of Bik and BAK and possibly other pro-apoptotic members of the Bcl2 family like Bad in bringing about apoptosis in the trigeminal ganglion.

It is possible that the different effects of BAX deficiency on apoptosis in trigeminal and SCG neurones deprived of NGF/TrkA signalling between E14 and P1 in vivo may not reflect an intrinsic difference in the function of BAX in these two kinds of neurones but might be related to the different stages of development over which these neurones were studied. Trigeminal neurones are born and undergo naturally occurring neuronal death at earlier developmental stages than SCG neurones. Whereas peak neurone number occurs in the trigeminal ganglion at E14 and neuronal death is largely over by birth (Davies and Lumsden, 1984), the peak in neurone number in the SCG occurs at E18 and neuronal death continues after birth (Wyatt et al., 1997; Francis et al., 1999). However, this seems unlikely because E18 BAX-deficient SCG neurones survive least 5 days in culture without NGF, whereas BAX-deficient trigeminal neurones cultured at the equivalent stage of development (from E14 embryos) die without NGF.

In addition to differences in the effects of BAX deficiency on apoptosis in different populations of NGF-dependent neurones deprived of NGF/TrkA signalling in vivo, we have also observed marked differences of BAX deficiency on neurone size in the absence of NGF/TrkA signalling. In the SCG of mice that lack both BAX and NGF/TrkA signalling, the neurones are much smaller than those of wild-type mice. This observation accords with the much reduced size of SCG neurones of BAX-deficient embryos grown without NGF in vitro (Deckwerth et al., 1996). Likewise, the DRG neurones are also much smaller in BAX-deficient neurones that lack NGF/TrkA signalling in vivo compared with wild-type mice (Patel et al., 2000). SCG neurones are also reduced in size in embryos that lack BAX alone, although not to the extent as in embryos that have defective NGF/TrkA signalling as well. The most parsimonious explanation for this observation is that elimination of neuronal death in the SCG of *Bax*<sup>-/-</sup> embryos means that there is a larger number of neurones competing for the same, limited supply of NGF. As a consequence, each neurone in *Bax*<sup>-/-</sup> embryos obtains less NGF than surviving

neurones would in wild-type mice, resulting in a reduction in the hypertrophic effects of NGF on the cell body. It is also possible that there may be some disruption of target field innervation in *Bax*<sup>-/-</sup> mice.

Surprisingly, the small number of neurones that remains in the trigeminal ganglia of mice lacking both BAX and either NGF or TrkA is much larger than those of wild-type mice. Indeed, many of these neurones are larger than the largest neurones observed in wild-type mice. As the trigeminal neurones of mice that lack any one of these proteins alone are not enlarged, this must mean that BAX-deficient trigeminal neurones grow in size when additionally deprived of NGF/TrkA signalling. Because mice that lack either NGF or TrkA die shortly after birth, it has not been possible to ascertain whether the enlarged neurones in the trigeminal ganglia of neonates lacking BAX and NGF or TrkA subsequently die. The reason for the enlargement is unclear, but may be a consequence of altered mitochondrial function or some kind of compensation, potentially via another Trk or neurotrophin family member.

In summary, we have shown that different populations of NGF-dependent neurones undergo cell death by either BAX-dependent or BAX-independent mechanisms when deprived for NGF/TrkA signalling in vivo. In future work it will be important not only to ascertain which pro-apoptotic members of the Bcl2 family are required for bringing about apoptosis in populations that die by a BAX-independent mechanism, but also to investigate whether different pro-apoptotic members of the Bcl2 family are involved in bringing about cell death in the same population of neurones exposed to different influences that cause cell death.

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