Timing of neuronal death in trkA, trkB and trkC mutant embryos reveals developmental changes in sensory neuron dependence on Trk signalling

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SUMMARY

The sensory neurons of the embryonic mouse trigeminal ganglion are supported in culture by different neurotrophins at successive stages of development. Initially the neurons survive in response to BDNF and NT3 and later switch to becoming NGF-dependent (Buchman, V. I. and Davies, A. M. (1993), Development 118, 989-1001). To determine if this in vitro switch in neurotrophin responsiveness is physiologically relevant, we studied the timing of neuronal death in the trigeminal ganglia of embryos that are homozygous for null mutations in the trkA, trkB and trkC genes, which encode receptor tyrosine kinases for NGF, BDNF and NT3, respectively. In wild-type embryos, the number of pyknotic nuclei increased from E11 to peak between E13 and E14, and decreased gradually at later ages, becoming negligible by birth. Neuronal death in the trigeminal ganglia of trkA⁻/⁻ embryos also peaked between E13 and E14, but was almost threefold greater than in wild-type embryos at this stage. Whereas there was no significant difference between the number of pyknotic nuclei in trkA⁻/⁻ and wild-type embryos at E11 and E12, there was a substantial increase in the number of pyknotic nuclei in the trigeminal ganglia of trkB⁻/⁻ at these earlier stages. Counts of the total number of neurons in E13 trigeminal ganglia revealed a marked decrease in trkB⁻/⁻ but not trkA⁻/⁻ or trkC⁻/⁻ embryos. Consistent with the later onset of excessive neuronal death in trkA⁻/⁻ embryos, there was a marked decrease in the neuronal complement of the trigeminal ganglia of trkA⁻/⁻ embryos at E15. These results demonstrate that TrkB signalling is required for the in vivo survival of many trigeminal neurons during the early stages of target field innervation before they become NGF-dependent.

Key words: neurotrophin, trk receptor tyrosine kinase, sensory neuron, cell death, mouse

INTRODUCTION

The neurotrophin family of proteins plays an important role in regulating neuronal survival in the developing vertebrate nervous system (Davies, 1994a). Four members of this family have been identified in mammals: nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT3) and NT4. These proteins exert their effects on neurons by binding to members of the trk family of receptor tyrosine kinases (Barbacid, 1994; Klein, 1994). Expression studies in cell lines have shown that TrkA is the receptor for NGF, TrkB is the receptor for BDNF and NT4, and TrkC is the receptor for NT3.

Developing sensory neurons survive in culture independently of neurotrophins at the stage when their axons are growing to their targets (Davies and Lumsden, 1984; Ernsberger and Rohrer, 1988; Vogel and Davies, 1991). The in vitro survival of many populations of cranial sensory neurons is initially promoted by BDNF or NT3 (Vogel and Davies, 1991; Buchman and Davies, 1993). Whereas several populations of cranial sensory neurons retain dependence on these neurotrophins in culture throughout the phase of naturally occurring neuronal death (Davies et al., 1986a,b; Hohn et al., 1990; Buj-Bello et al., 1994), other populations switch dependence to NGF during the early stages of target field innervation (Buchman and Davies, 1993; Buj-Bello et al., 1994).

The in vitro switch from BDNF/NT3 dependence to NGF dependence has been studied most extensively in the trigeminal ganglion neurons of the mouse embryo (Buchman and Davies, 1993; Paul and Davies, 1995), but also occurs in cultured jugular ganglion neurons (Davies et al., 1993a; Buj-Bello et al., 1994). When grown at low density in defined medium, E10 mouse trigeminal neurons, which have not yet innervated their peripheral targets, die between 24 hours and 48 hours in the absence of neurotrophins. The death of these early neurons is prevented by BDNF or NT3 but not by NGF. NGF promotes the survival of a small proportion of E11 neurons, and virtually all E12 neurons, for 48 hours in culture. Concomitant with acquisition of the NGF survival response, the survival responses to BDNF and NT3 are lost. In E13 cultures, less than 10% of the neurons are supported for 48 hours by BDNF, and the survival response to NT3 falls to a similar level in E14 cultures. It is not known, however, if this apparent switch in the survival response of developing trigeminal neurons from BDNF/NT3 to NGF observed in culture takes place in vivo during normal development.
Analyses of mice that have targeted null mutations in the neurotrophin genes (Crowley et al., 1994; Ernfors et al., 1994a,b; Farinas et al., 1994; Jones et al., 1994; Conover et al., 1995; Liu et al., 1995) and trk genes (Klein et al., 1993, 1994; Smye et al., 1994) have been extremely useful for assessing the physiological relevance of earlier in vitro studies of the effects of neurotrophins on neuronal survival, and for extending our understanding of the functions of neurotrophins in neuronal development. Several studies of mutant mice suggest that BDNF and NT3 play a role in trigeminal ganglion development. By birth and the postnatal period there are marked reductions in the neuronal complement of the trigeminal ganglia of BDNF−/− (Ernfors et al., 1994a; Jones et al., 1994), trkB−/− (Klein et al., 1993) and NT3−/− mice (Ernfors et al., 1994b). However, because trigeminal ganglia were not studied at intervals throughout embryonic development, it is not known when BDNF and NT3 are required for neuron survival or whether they influence the neuronal complement of the ganglion by promoting precursor cell proliferation and/or differentiation. The latter possibilities are raised by the findings that BDNF directs pluripotent neural crest cells to differentiate in vitro along the sensory neuron lineage (Sieber-Blum, 1991), and that NT3 is a mitogen for cultured neural crest cells (Kalcheim et al., 1992) and enhances the proliferation or differentiation of sensory neuron progenitor cells in culture (Wright et al., 1992).

To resolve the above issues, we have quantified the extent of neuronal death in the trigeminal ganglia of trkA−/−, trkB−/−, trkC−/− and wild-type mice at closely staged intervals throughout embryonic development. Our results demonstrate that neurotrophin switching takes place during embryogenesis and have clarified the role of Trk receptor tyrosine kinases in sensory neuronal development by providing precise data on the timing of neuronal dependence on Trk signalling in vivo.

MATERIALS AND METHODS

Animals

Embryos were obtained from overnight matings of (i) trkA+/+ mice, (ii) trkB−/− and (iii) trkC−/− mice in a 129·m background. Pregnant females were killed by cervical dislocation at the required stage of gestation and the precise stage of development was determined by the criteria of Theiler (Theiler, 1972). The genotypes of the embryos were determined by PCR using DNA isolated from embryonic tissues (Schimmang et al., 1995).

Quantification of numbers of pyknotic nuclei in the trigeminal ganglion

Embryos were fixed by immersion in 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.3. The brain was removed from E14 and older embryos to allow proper fixation of the trigeminal ganglia. After fixation for at least a week, the embryos were dehydrated and embedded in paraffin wax. E15 and older 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 fourth section along the entire rostrocaudal extent of the trigeminal ganglia of E11 to E19 embryos. Estimates of the total number of pyknotic nuclei in each ganglion were obtained by multiplying the sum of these counts by four. At each age, between four and ten ganglia of the following genotypes were studied: trkA+/−, trkB−/−, trkC−/− and wild type mice. In all cases, the sections were coded prior to counting to avoid any observer bias.

Quantification of total numbers of neurons in the trigeminal ganglion

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 mm2 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). The estimates of the total number of neurons in the trigeminal ganglion of normal embryos in the present study are very similar to those previously obtained (Davies and Lumsden, 1984) by counting neuronal nuclei and correcting for split nuclei (Abercrombie, 1946).

Immunohistochemistry

To identify the proportion of neurons undergoing cell death in the early trigeminal ganglia of wild-type and trkB−/− embryos, 8 μm frozen sections were double labelled for neuron-specific markers (peripherin or 160 kDa neurofilament protein) and 3′ DNA ends generated by DNA fragmentation in apoptotic cells. Sections were incubated with either rabbit anti-peripherin antibody (Chemicon) diluted 1:300 in phosphate-buffered saline containing 0.1% Triton and 1% goat serum for 12 hours, or mouse anti-160 kDa monoclonal antibody (Sigma) diluted 1:200 in phosphate-buffered saline containing 0.3% Triton and 1% horse serum for 48 hours. These primary antibodies were localised by an immunoperoxidase technique using a biotinylated secondary antibody and an avidin/biotinylated horse radish peroxidase marcomolecular complex (Vectastain ABC Kit, Vector Laboratories). This was followed by in situ DNA end-labelling with fluorescein-labelled nucleotides (ApopTag Direct Kit, Oncor). The sections were examined and photographed using a Axioskop microscope. Cells undergoing apoptosis were recognised by an intensely fluorescent nucleus. Apoptotic cells were classified as neurons if a peroxidase-labelled rim of cytoplasm was clearly visible.

RESULTS

Developmental changes in the number of pyknotic nuclei

Pyknotic nuclei in developing trigeminal ganglia were recognised as one or more very darkly stained spherical structures containing within a clearly visible membrane (Fig. 1). The great majority of these pyknotic nuclei were observed in large degenerating cells, suggesting that these were neurons (Oppenheim, 1991). In late fetal ganglia, a very small number of pyknotic nuclei were also observed in very small cells that were of a similar size to satellite cells, suggesting that a minority of the supporting cells undergo cell death in the ganglion. These latter pyknotic cells were not counted.

(i) Wild-type embryos

In wild-type embryos, pyknotic nuclei were observed as early as E11. The number increased steadily by fivefold to reach a
peak at E14, after which there was a decrease to a negligible number by the end of fetal development at E19 (Fig. 2).

(ii) \textit{trkA}^{-/-} embryos

The number of pyknotic nuclei in the trigeminal ganglion of \textit{trkA}^{-/-} embryos was very similar to that of wild-type embryos at E11 and E12 (Fig. 2). In contrast to wild-type embryos, there was a marked increase in the number of pyknotic nuclei between E12 and E13. At E13 and E14 the number of pyknotic nuclei in the trigeminal ganglia of \textit{trkA}^{-/-} embryos was approximately threefold greater than in ganglia of wild-type embryos. After E14 there was a marked fall in the number of pyknotic nuclei to less than in wild-type ganglia at E15. The number of pyknotic nuclei fell further to the same negligible level by just before birth (E19), as in wild-type embryos. There was no obvious difference in the appearance of pyknotic nuclei in the trigeminal ganglia of \textit{trkA}^{-/-} embryos and wild-type embryos at equivalent stages of development.

(iii) \textit{trkB}^{+/-} embryos

The number of pyknotic nuclei in the trigeminal ganglion of \textit{trkB}^{+/-} embryos was substantially higher than that of wild-type embryos at E11 and E12 (Fig. 2). There were over fourfold more pyknotic nuclei at E11 and over threefold at E12. From a clear peak in the number of pyknotic nuclei in \textit{trkB}^{+/-} ganglia at E12 there was a marked fall to levels less than in wild-type ganglia at E13. At E15 and later ages, the number of pyknotic nuclei in \textit{trkB}^{+/-} ganglia was similar to that in wild-type ganglia. There was no obvious difference in the appearance of pyknotic nuclei in the trigeminal ganglia of \textit{trkB}^{+/-} embryos and wild-type embryos at equivalent stages of development.

(iv) \textit{trkC}^{-/-} embryos

There was a small increase in the number of pyknotic nuclei in the early trigeminal ganglia of \textit{trkC}^{-/-} embryos (Fig. 2). The number of pyknotic neurons was just over twofold greater than in wild-type ganglia at E11, and was 1.5-fold greater at E12. Although these increases in pyknotic nuclei in \textit{trkC}^{-/-} ganglia were far less than in \textit{trkB}^{+/-} ganglia, they were nonetheless statistically significant (\textit{t}-tests: E11, \textit{P}<0.001; E12, \textit{P}<0.02). At

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**Fig. 2.** Graph comparing the number pyknotic nuclei in the trigeminal ganglia of wild-type, \textit{trkA}^{-/-}, \textit{trkB}^{+/-} and \textit{trkC}^{-/-} embryos from E11 to E19. The data were obtained from 4-10 ganglia of wild-type embryos and 4-6 ganglia of \textit{trkA}^{-/-} embryos at each age (data were obtained from at least three separate embryos of each kind at each age). The means and standard errors of the mean are shown.

E13 and later ages there were similar numbers of pyknotic nuclei in \textit{trkC}^{-/-} and wild-type ganglia. As in \textit{trkA}^{-/-} and \textit{trkB}^{+/-} ganglia, there were no obvious differences in the appearance of pyknotic nuclei in the trigeminal ganglia of \textit{trkC}^{-/-} embryos and wild-type embryos at equivalent stages of development.

**Differences in the neuronal complement of the trigeminal ganglion of wild-type and mutant embryos at key stages of development**

To ascertain the consequences of the differences in the number of dying cells in the trigeminal ganglia of wild-type and mutant embryos at different stages of development, we estimated the total number of neurons in the ganglion at several key stages (Fig. 3). At E13, when the total number of neurons in the trigeminal ganglion reaches a peak during normal development (Davies and Lumsden, 1984), there were no statistically significant differences in the total numbers of neurons in the trigeminal ganglia of wild-type, \textit{trkA}^{-/-} and \textit{trkC}^{-/-} embryos (\textit{t}-tests: \textit{P}>0.1). In contrast, there was a statistically significant, 42% reduction in the number of neurons in the ganglia of \textit{trkB}^{+/-} embryos at this stage of development (\textit{t}-test: \textit{P}<0.001). At E15, shortly after the number of neurons in the trigeminal ganglion begins to decline in normal development (Davies and Lumsden, 1984), there was a marked, statistically significant, 64% reduction in the number of neurons in \textit{trkC}^{-/-} ganglia compared with wild-type ganglia (\textit{t}-test: \textit{P}<0.001). The number of neurons in E15 \textit{trkB}^{+/-} ganglia was significantly reduced by 33% compared with wild-type ganglia (\textit{t}-test, \textit{P}<0.01). Although there was a small reduction in the number of neurons in E15 \textit{trkC}^{-/-} ganglia compared with wild-type ganglia, this was not statistically significant (\textit{t}-test: \textit{P}>0.1). By E17, close to the end of the phase of naturally occurring
neuronal death, the reduction in the number of neurons in trkA-/- ganglia had further increased to 69% compared with wild-type ganglia. The number of neurons in E17 trkB-/- ganglia was still significantly reduced by 33% compared with wild-type ganglia (t-test, P<0.0005). By E17, however, there was a 22% reduction in the number of neurons in the ganglia of trkC-/- embryos compared with wild-type embryos that was just within the limits of significance (t-test, P<0.05).

The majority of cells dying in early trkB-/- ganglia are neurons

To determine if the increased cell death in early trkB-/- trigeminal ganglia was due to increased loss of neurons, sections of trkB-/- and wild-type embryos were double labelled for neuron markers and apoptotic nuclei at the peak of cell death in trkB-/- trigeminal ganglia at E12. Apoptotic cells were recognised by intense nuclear fluorescence following in situ DNA end-labelling with fluorescein-labelled nucleotides. Neurons were positively identified by cytoplasmic staining for either 160 kDa neurofilament protein or peripherin, using an immunoperoxidase technique. All of the apoptotic cells in several sections of each ganglion were scored, as either neurons or other cells. An apoptotic cell was scored as a neuron if the fluorescent nuclear staining was clearly and unambiguously contained within a distinct rim of peroxidase-labelled cytoplasm (Fig. 4A). There was no significant difference between the proportion of apoptotic cells positively identified as neurons by neurofilament staining in E12 wild-type embryos (67.3±3.6%, n=3 ganglia from different embryos) and E12 trkB-/- embryos (69.0±1.9%, n=3). Likewise, there was no significant difference between the proportion of apoptotic cells positively identified as neurons by peripherin staining in E12 wild-type embryos (70.0±3.7%, n=3) and E12 trkB-/- embryos (71.6±5.6%, n=3). These results show that the majority of dying cells in early wild-type and trkB-/- trigeminal ganglia are neurons. However, because of the strict criteria for classifying apoptotic cells as neurons, the actual proportion of dying cells that are neurons is likely to be even greater than our estimates suggest. Fig. 5 illustrates the large difference in the numbers of apoptotic cells between wild-type and trkB-/- ganglia at E12.

DISCUSSION

Several of our findings suggest that many trigeminal neurons require functional TrkB receptors for survival at an early stage in their development. First, the number of pyknotic nuclei in E11 and E12 ganglia was substantially greater in trkB-/- embryos compared with wild-type embryos. Second, the majority of cells dying in early trkB-/- ganglia were positively identified as neurons. Third, the total number of neurons in the trigeminal ganglia of trkB-/- embryos was substantially reduced at E13 compared with wild-type embryos. TrkB is the preferred receptor for two neurotrophins, BDNF (Glass et al., 1991; Klein et al., 1991b; Soppet et al., 1991; Squinto et al., 1991) and NT4 (Berkemeier et al., 1991; Ip et al., 1992; Klein et al., 1992). Previous in vitro studies have shown that both BDNF and NT4 promote the survival of the majority of embryonic mouse trigeminal ganglion neurons at an early stage in their development and that the majority of neurons lose responsiveness to these factors by E13 (Buchman and Davies, 1993; Davies et al., 1993a; Paul and Davies, 1995). The finding that postnatal BDNF-/- mice (Emfors et al., 1994a; Jones et al., 1994), but not NT4-/- mice (Conover et al., 1995; Liu et al., 1995), have marked reductions of the numbers of neurons in the trigeminal ganglion, suggests that BDNF, not NT4, is the physiologically relevant TrkB ligand for developing trigeminal ganglion neurons. However, because NT3 can also promote the survival of embryonic trigeminal neurons by signalling via TrkB (Davies et al., 1995), we cannot exclude the possibility that NT3 acting via TrkB is also important for the early survival of trigeminal neurons in vivo.

Our study has demonstrated that trigeminal neurons depend on functional TrkA receptors for survival at a later stage of development than their dependence on TrkB signalling. At E11 and E12, when there are substantially more dying cells in the trigeminal ganglia of trkB-/- embryos than in wild-type embryos, there are normal numbers of dying cells in the trigeminal ganglia of trkA-/- embryos. Accordingly, at E13, there is no significant difference between the total number of neurons in the trigeminal ganglia of trkA-/- and wild-type embryos, whereas there is a marked reduction in the neuronal complement in the ganglion of trkB-/- embryos by this stage. At E13 and E14, there are substantially higher numbers of dying neurons in the trigeminal ganglion of trkA-/- embryos than wild-type embryos, and by E15 there is a marked reduction in the neuronal complement of the trkA-/- ganglion. The later onset of survival dependence of trigeminal neurons on TrkA signalling in vivo is consistent with the later acquisition of the survival response of cultured trigeminal neurons to NGF (Buchman and Davies, 1993), the preferred TrkA ligand (Hempstead et al., 1991; Kaplan et al., 1991; Klein et al., 1991a). Our findings therefore provide formal genetic proof for the switch in survival dependence of early trigeminal ganglion neurons from TrkB to TrkA ligands.
Estimates of the number of neurons in the trigeminal ganglion towards the end of the phase of naturally occurring neuronal death at E17 indicate that during embryonic development TrkA signalling is required for the survival of more trigeminal neurons than TrkB signalling. This in turn suggests that not all trigeminal neurons depend on TrkB for survival before becoming NGF-dependent. Because our data suggest that TrkB signalling is important for the survival of many neurons during the early stages of trigeminal ganglion development (at E11 and E12), it is possible that only early-born trigeminal neurons depend sequentially on TrkB and TrkA signalling for survival. Although detailed information on the time-course of neuronal differentiation in the mouse trigeminal ganglion is not available, the demonstration that the number of trigeminal neurons increases in the ganglion to E13 (Davies and Lumsden, 1984) and that new axons are recruited to the trigeminal nerve up to this time (Davies, 1987) suggests that trigeminal neurons are born until at least E13. The transient dependence of early-born trigeminal neurons on TrkB ligands has been proposed to sustain the survival of many of these neurons until the majority of later-born neurons have extended axons to their targets, so that most neurons would compete for target-derived NGF during the same period of development (Davies, 1994b).

Although we have observed a small, statistically significant increase in the number of pyknotic nuclei in the trigeminal ganglia of trkC−/− embryos at E11 and E12, there was no significant reduction in the total number of neurons in the trigeminal ganglia of trkC−/− embryos at E13. This is surprising given the early survival response of cultured trigeminal to NT3 (Buchman and Davies, 1993; Paul and Davies, 1995) and the expression of trkC mRNA by many neurons in the early trigeminal ganglia of the rat embryo (Ernfors et al., 1992). Furthermore, there is a 61-64% reduction in the number of neurons
in the trigeminal ganglia of postnatal NT3<sup>−/−</sup> mice (Ernfors et al., 1994b; Farinas et al., 1994), and a recent analysis of the trigeminal ganglion of NT3<sup>−/−</sup> embryos suggests that NT3 acts early in trigeminal ganglion development to promote the survival of proliferating trigeminal neuron precursor cells (Elishamy and Ernfors, 1996). These findings suggest that, whereas NT3 plays an important role in the early development of the trigeminal ganglion, TrkC tyrosine kinase receptor does not play a major role in mediating the effects of NT3 at this stage.

Although there was no significant reduction in the neuronal complement of the trkC<sup>−/−</sup> ganglion at E13, we observed a small, statistically significant reduction in the number of neurons in the trigeminal ganglion of E17 trkC<sup>−/−</sup> embryos compared with wild type, suggesting that a small proportion of fetal trigeminal neurons depends on TrkC signalling for survival. It is known that a small proportion of neurons are supported in cultures of late fetal trigeminal cultures by low concentrations of NT3 and that these neurons are larger than those that are supported by NGF (Davies et al., 1993b, 1995). Thus, it is possible that this small, distinctive subset of neurons is lost in the trkC<sup>−/−</sup> ganglion.

Previous studies have also revealed differences between the phenotypes of trkC<sup>−/−</sup> and NT3<sup>−/−</sup> mice. There is a 55-78% reduction in the number of neurons in the lumbar DRG of NT3<sup>−/−</sup> neonates (Ernfors et al., 1994b; Farinas et al., 1994) compared with only a 17-19% reduction in the lumbar DRG of trkC<sup>−/−</sup> neonates (Klein et al., 1994; Minichiello et al., 1995). Likewise, the neuronal complement of the spiral ganglion of NT3<sup>−/−</sup> neonates is reduced by 85% at E5 to 87% (Farinas et al., 1994; Ernfors et al., 1995) compared with a 51% reduction in trkC<sup>−/−</sup> neonates (Schimmang et al., 1995). These differences are likely to be due to the ability of NT3 to signal via TrkA and TrkB in embryonic sensory neurons (Davies et al., 1995). NT3 is able to promote the in vitro survival of the majority of trigeminal and nodose neurons obtained from E14 trkC<sup>−/−</sup> embryos but not from embryos that also lack functional TrkA or TrkB receptors. Interestingly, the ability of NT3 to signal via TrkA and TrkB in sensory neurons decreases during development, becoming negligible by E18 (Davies et al., 1995). Thus, it is possible that NT3 promotes the survival of trigeminal neuron progenitor cells by acting at least in part via non-preferred trk receptor tyrosine kinases.

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