Development 120, 1573-1580 (1994)
Printed in Great Britain © The Company of Biologists Limited 1994

The survival of NGF-dependent but not BDNF-dependent cranial sensory neurons is promoted by several different neurotrophins early in their development

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SUMMARY

Recent work has shown that the survival of the nerve growth factor (NGF)-dependent trigeminal ganglion neurons of the mouse embryo is promoted by brain-derived neurotrophic factor (BDNF) and neurotrophin-3 (NT-3) during the early stages of target field innervation (Buchman and Davies, (1993) *Development,* 118, 989-1001). The present study was undertaken to ascertain if responsiveness to multiple neurotrophins is a universal feature of the early stages of neuronal development or is restricted to only certain kinds of neurons. To address this issue, we took advantage of the accessibility, from an early developmental stage, of several populations of cranial sensory neurons in the chicken embryo that depend for survival on just one or two known neurotrophins during the phase of naturally occurring cell death. During the mid-embryonic period (E10 to E12) when the number of sensory neurons is declining due to naturally occurring neuronal death, the neurons of the jugular ganglion and the dorsomedial part of the trigeminal ganglion (DMTG) were supported by NGF, the neurons of the ventrolateral part of the trigeminal ganglion (VLTG) were supported by BDNF and the nodose ganglion contained a major subset of neurons supported by BDNF and a minor subset supported by NT-3. Earlier in development (E6), the survival of DMTG and jugular neurons was additionally promoted by BDNF and NT-3. In contrast, E6 VLTG neurons did not exhibit a survival response to either NGF or NT-3, and E6 nodose neurons did not exhibit a survival response to NGF. The loss of the early survival response of DMTG and jugular neurons to BDNF and NT-3 was due to a marked shift in the dose-response to these neurotrophins (by more than four orders of magnitude between E6 and E8). These findings indicate that the responsiveness of certain populations of neurons to neurotrophins becomes restricted during development and raise the possibility that neuron-target interactions involving multiple neurotrophins regulate neuronal survival in vivo.

Key words: trigeminal ganglion, nodose ganglion, neurotrophins, NGF, BDNF, NT-3

INTRODUCTION

A family of homologous proteins termed neurotrophins promotes the survival of neurons in the developing vertebrate nervous system. To date, five members of this family have been identified: nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), *Xenopus* neurotrophin-4 (NT-4) and mammalian neurotrophin-4/5 (NT-4/5)(Levi-Montalcini and Angeletti, 1968; Barde et al., 1982; Leibrock et al., 1989; Hohn et al., 1990; Rosenthal et al., 1990; Maisonpierre et al., 1990; Ernfors et al., 1990; Jones and Reichardt, 1990; Hallbook et al., 1991; Berkemeier et al., 1991; Ip et al., 1992). The available evidence suggests that each neurotrophin promotes the survival of a particular set of developing neurons. For example, whereas NGF supports sympathetic neurons (Greene, 1977), neural crest-derived cutaneous sensory neurons (Davies and Lindsay, 1985) and basal forebrain cholinergic neurons (Hatanaka et al., 1988), BDNF supports proprioceptive, enteroceptive and placode-derived cutaneous sensory neurons (Davies et al., 1986a,b) and the dopaminergic neurons of the substantia nigra (Hyman et al., 1991).

Cranial sensory neurons have been especially useful for studying the specificity and time-course of the survival-promoting effects of neurotrophins (Davies, 1987). These neurons can be obtained for experimental studies from the earliest stages of their development, and in contrast to the functional heterogeneity of the sensory neurons in dorsal root ganglia (DRG), cranial sensory neurons are segregated into functionally distinct populations. Perhaps as a consequence of this functional segregation, different populations of cranial sensory neurons have distinct neurotrophin requirements when cultured during the period of naturally occurring cell death. For example, the small-diameter cutaneous sensory neurons of the jugular ganglion and the dorsomedial part of the trigeminal ganglion (DMTG) are supported by NGF (Davies and Lumsden, 1983; Davies and Lindsay, 1985) but show little response to BDNF (Davies et al., 1986b) or NT-3 (Hohn et al.,

1990). The large-diameter cutaneous sensory neurons of the ventrolateral part of the trigeminal ganglion (VLTG) are supported by BDNF (Davies et al., 1986b) but show little response to NGF (Davies and Lumsden, 1983) or NT-3 (Hohn et al., 1990). The proprioceptive neurons of the trigeminal mesencephalic nucleus (TMN) are supported by BDNF or NT-3 (Davies et al., 1986a; Hohn et al., 1990) but are unresponsive to NGF (Davies et al., 1987). The enteroceptive neurons of the nodose ganglion contain a major subpopulation of BDNF-dependent neurons and a minor subpopulation of NT-3-dependent neurons (Lindsay et al., 1985; Davies et al., 1986b; Hohn et al., 1990), but are unresponsive to NGF (Lindsay and Rohrer, 1985).

Developmental studies have shown that cranial sensory neurons survive independently of neurotrophins when their axons are growing to their targets and acquire neurotrophin dependence close to the time their axons reach their targets (Davies and Lumsden, 1984; Vogel and Davies, 1991). In a recent study of the survival-promoting effects of different neurotrophins on embryonic mouse trigeminal neurons, a switch from responsiveness to BDNF and NT-3 to responsiveness to NGF was observed to take place during the early stages of target field innervation (Buchman and Davies, 1993). When trigeminal axons come into proximity with their peripheral targets, the neurons become responsive to BDNF and NT-3. Shortly afterwards, the neurons become additionally responsive to NGF. With the onset of cell death in the trigeminal ganglion, the neurons become refractory to BDNF and NT-3 but remain dependent on NGF for survival throughout the phase of naturally occurring cell death. The present study was undertaken to ascertain if responsiveness to multiple neurotrophins is a universal feature of the early stages of neuronal development, and to provide information on how early neurons selectively lose responsiveness to certain neurotrophins. From studying several populations of avian cranial sensory neurons at stages throughout their early development, we show that only neurons that depend on NGF for survival throughout the phase of naturally occurring cell death respond to multiple neurotrophins earlier in development. In contrast, neurons that depend on BDNF for survival throughout the phase of naturally occurring cell are restricted in their response to this factor during earlier developmental stages.

MATERIALS AND METHODS

White Leghorn chicken eggs were incubated at 38°C in a forced-draft incubator until the required age. The trigeminal, jugular and nodose ganglia were dissected from chicken embryos of 6, 8, 10 and 12 days incubation: stages 29, 34, 36 and 38, respectively (Hamburger and Hamilton, 1951). The trigeminal ganglion was sub-dissected (Davies and Lumsden, 1983) into its neural crest-derived dorsomedial pole (DMTG) and placode-derived ventrolateral pole (VLTG). The median component of the trigeminal mesencephalic nucleus (TMN) was dissected from E10 embryos (Davies, 1986).

After incubating in 0.1% trypsin in calcium- and magnesium-free Hank's balanced salt solution (HBBS) for 12 minutes at 37°C, the dissected tissue was washed twice in Hank's F12 medium plus 10% heat-inactivated horse serum (HIHS) and was dissociated by gentle trituration using a fire-polished Pasteur pipette. Non-neuronal cells were removed from E8 and older neurons by differential sedimentation (Davies, 1986). The resulting pure neuronal suspensions and the dissociated cell suspensions from E6 ganglia (at which age differential sedimentation is not very effective in removing non-neuronal cells) were plated at very low density in 35 mm tissue culture dishes (250-500 per dish) which had been coated with polyornithine (0.5 mg/ml, Sigma P8638, overnight) and laminin (20 μg/ml, Gibco-BRL, 4 hours). The cells were grown in 2 ml of Ham's F14 medium supplemented with 10% HIHS at 37°C. For experiments in which very high concentrations of neurotrophins were used, the neurons were grown in the 11 mm diameter wells of 4-well dishes (Greiner) that had been pre-coated with polyornithine and laminin. This permitted us to use only 100 μl of culture medium in each well. Purified NGF (gift of W. Mobley) and purified recombinant BDNF and NT-3 (gifts of Gene Burton and John Winslow, Genentech Inc.) were added to these cultures either alone or in combination.

To assess the proportion of neurons that survive in control cultures (no added neurotrophins) and in the presence of different neurotrophins, the number of neurons within a 144 mm^2 grid was counted 6 hours after plating. The number of surviving, process-bearing neurons was subsequently counted 48 hours after plating (by which time almost all neurons had degenerated in control cultures). This latter number was expressed as a percentage of the initial number of neurons at 6 hours. In each experiment, control and neurotrophin-supplemented cultures were set up in triplicate. Each experiment was repeated at least twice. The same procedure was used to assess the proportion of neurons surviving in cultures set up in 4-well dishes, except that the entire surface of the wells was examined for neurons.

To ascertain if the increased number of neurons observed in E6 cultures in the presence of neurotrophins was due to a direct survival response of neurons (as opposed to the differentiation of neuron progenitor cells present in these cultures), the fate of individual neurons in low density dissociated cultures was followed (Vogel and Davies, 1991). This was done by recording the location, 12 hours after plating, of unambiguously identifiable bipolar neurons within a 9×9 mm grid, scored on the under surface of polyornithine/laminin-coated 60 mm culture dishes and monitoring at 12-hourly intervals until 48 hours if each of these neurons was surviving or had degenerated. To avoid any possible effects of neurotrophins on the size of the initial cohorts, these factors were added after the identification of cohorts at 12 hours.

RESULTS

Differences and developmental changes in the response of cranial sensory neurons to neurotrophins

By 48 hours incubation, the great majority of neurons in control cultures of DMTG, jugular, VLTG and nodose neurons had degenerated. This permitted the survival response of these neurons to NGF, BDNF and NT-3 to be clearly observed at this time. The graphs in Fig. 1 show the magnitude and age-related changes in the survival-promoting effects of neurotrophins on these neurons from E6 to E12. The bar charts in Fig. 2 compare the survival of neurons grown with each neurotrophin alone with the survival of neurons grown in the presence of different combinations of neurotrophins. Where two neurotrophins promote the survival of neurons in a given population, comparison of the numbers of neurons surviving in the presence of each neurotrophin alone with the number surviving in the presence of both neurotrophins will reveal whether the neurotrophins support the same or different subsets of neurons in the population.

DMTG neurons

Fig. 1 shows that the survival of the majority of DMTG neurons was promoted by NGF throughout the E6 to E12 age

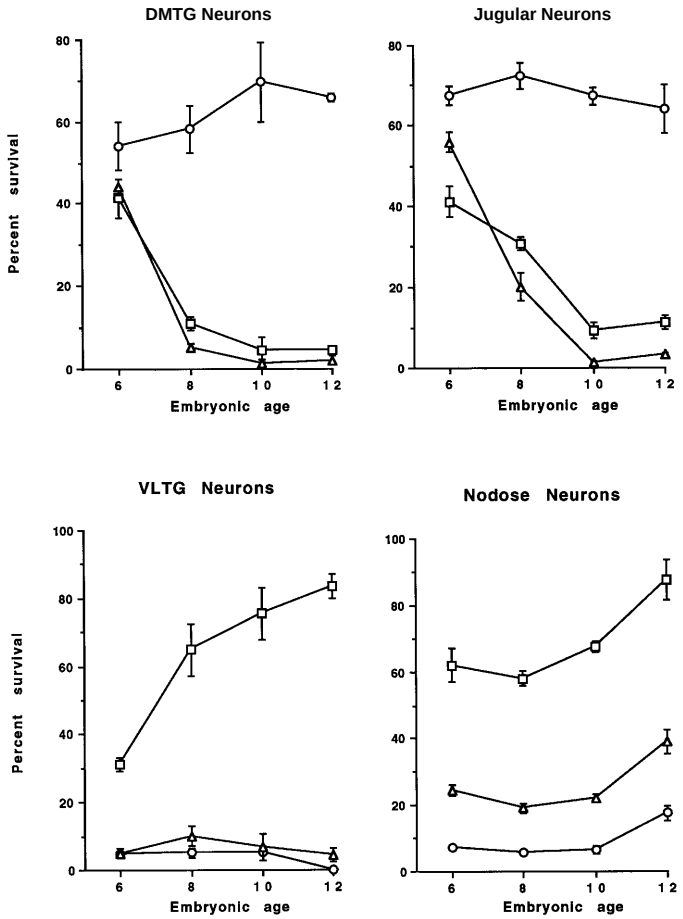

Fig. 1. Age-related changes in the survival responses of cranial sensory neurons to neurotrophins. Graphs showing the percentage survival of DMTG, jugular, VLTG and nodose neurons from E6 to E12 embryos after 48 hours incubation with 2 ng/ml of NGF (circles), BDNF (squares) or NT-3 (triangles). The mean ± the standard error are shown (*n*=3).

range. In addition, at E6, almost as many neurons were supported by either BDNF or NT-3. This early response to BDNF and NT-3 was, however, short-lived. There was a marked fall in the number of neurons surviving in the presence of BDNF or NT-3 between E6 and E8, and by E12 less than 5% of the neurons were supported by these factors, compared with over 60% supported by NGF.

Fig. 2 shows that when E6 DMTG neurons were grown in the presence of NGF plus BDNF or NGF plus NT-3, there were very few additional surviving neurons compared with cultures containing NGF alone. Likewise, there was negligible additional neuronal survival in cultures containing BDNF plus NT-3 compared with cultures containing either of these neurotrophins alone. These results indicate that there are not separate subpopulations of neurons that respond to each neurotrophin at E6, rather, the majority of E6 neurons respond to all three neurotrophins.

Jugular neurons

As with DMTG neurons, the survival of the majority of jugular neurons was promoted by NGF at all ages. Jugular neurons also showed a marked, transitory survival response to BDNF and NT-3 at E6. The number of neurons surviving in the presence of BDNF or NT-3 decreased markedly at later ages, reaching 10% or less by E10 (Fig. 1).

There was negligible additional neuronal survival in E6 cultures containing NGF plus BDNF or NGF plus NT-3 compared with cultures containing NGF alone, indicating that at this early stage the majority of neurons supported by NGF also respond to BDNF and NT-3. Likewise, there was negligible additional neuronal survival in E6 cultures containing BDNF plus NT-3 compared with cultures containing either neurotrophin alone (Fig. 2).

VLTG neurons

At all ages, the survival of VLTG neurons was supported by BDNF. This response to BDNF increased from E6 to E8, after which it remained in the region of 70-80% survival. In marked contrast to the early response of DMTG and jugular neurons to all three neurotrophins, only a small percentage of VLTG neurons were supported by NGF or NT-3 (less than 10% at any age) and there were no age-related changes in this response (Fig. 1). The percentage survival of VLTG neurons in the presence of BDNF was not significantly changed by the addition of either NGF or NT-3 to the culture medium (Fig. 2).

Because many VLTG neurons are generated before DMTG neurons (D'Amico-Martel and Noden, 1980), it is possible that VLTG neurons may have passed through a period of responsiveness to multiple neurotrophins before E6, the age at which DMTG neurons respond to NGF, BDNF and NT-3. To exclude this possibility, we cultured VLTG neurons with different neurotrophins at E5. After 48 hours incubation 9.0±0.8% of the neurons were surviving in control cultures and there were no significant differences in the number of neurons surviving in the presence of either NGF (8.5±0%) or NT-3 (13.0±5.9%). There was, however, a clear survival response to BDNF (36.4±2.5%). Thus, in contrast to early DMTG neurons, VLTG neurons at an equivalent early stage in their development do not exhibit responsiveness to multiple neurotrophins.

Nodose neurons

Throughout the period studied, the survival of nodose neurons was supported by BDNF and NT-3. Of the two, BDNF was more effective (60% to over 80% of the neurons surviving with this factor compared with 20-40% with NT-3. At all ages, the numbers of neurons surviving in the presence of NGF was not significantly greater than the numbers of neurons in control cultures (Fig. 2). Almost all neurons survived in the presence of both BDNF and NT-3 (Fig. 2), indicating that the nodose ganglion comprises two partially overlapping populations of BDNF-responsive and NT-3-responsive neurons.

BDNF and NT-3 exert a direct survival response from early DMTG and jugular neurons

Because the neural crest-derived DMTG and jugular neurons are born between E4 and E6 in vivo (D'Amico-Martel, 1982), cell cultures set up at E6 may contain neuron progenitor cells that are capable of differentiating into neurons in vitro (Rohrer et al., 1985). Thus, it was important to ascertain if the increase in the number of neurons in these cultures in the presence of BDNF or NT-3 was due to a survival response of differentiated neurons or was due to enhanced proliferation or differentiation of progenitor cells. This is especially pertinent because BDNF may direct pluripotent neural crest cells to differentiate

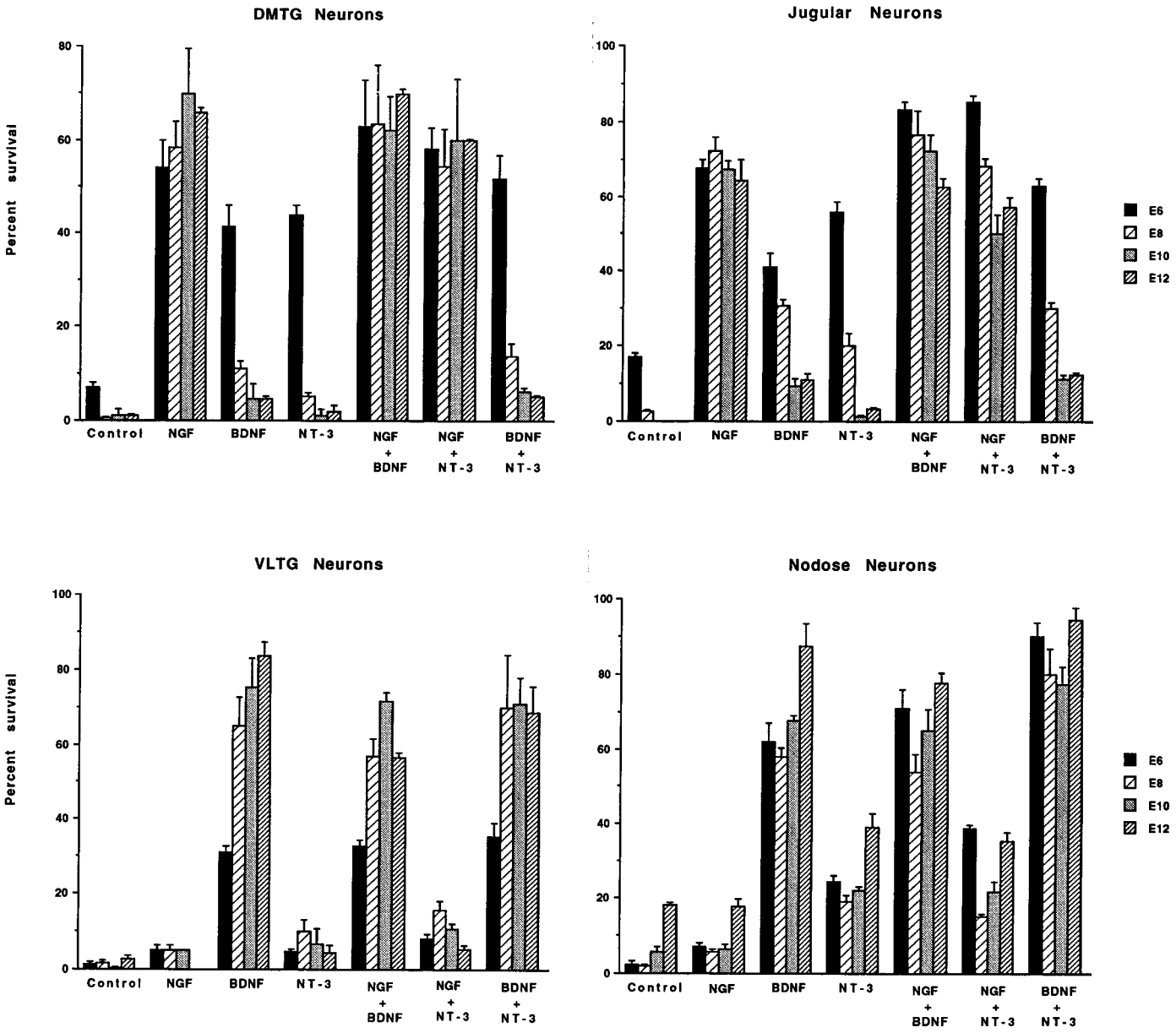

Fig. 2. Survival responses of cranial sensory neurons to neurotrophins alone and in combination. Bar charts showing the percentage survival of DMTG, jugular, VLTG and nodose neurons from E6 (black bars), E8 (light hatched bars), E10 (stippled bars) and E12 (dark hatched bars) embryos after 48 hours incubation with 2 ng/ml of NGF, BDNF, NT-3, NGF + BDNF, NGF + NT-3 or BDNF + NT-3. The mean ± the standard error are shown (n=3).

along the sensory neuron lineage (Sieber-Blum, 1991) and NT-3 increases the proliferation of neural crest cells (Kalcheim et al., 1992) and enhances the proliferation or differentiation of dorsal root ganglion neuron progenitor cells (Wright et al., 1992).

Fig. 3 shows the percentage survival after 48 hours incubation in cohorts of E6 DMTG neurons identified 6 hours after plating. The majority of neurons in these cohorts were still surviving in the presence of either BDNF or NT-3, whereas only 10% were alive in control cultures. Moreover, the percentage survival in control and neurotrophin-supplemented neuronal cohorts was very similar to the overall percentage survival in E6 DMTG cultures in which the fate of individual neurons was not serially monitored (Fig. 1). These results demonstrate that the effect of BDNF and NT-3 on the number of neurons in E6 DMTG cultures is due mainly, if not exclusively, to enhanced survival. A smaller number of cohort experiments carried out with E6 jugular neurons gave similar results (data not shown).

Dose responses of DMTG neurons to neurotrophins shift to higher concentrations with age

The loss of the early survival response of DMTG and jugular neurons to BDNF and NT-3 could be due either to increasing numbers of the neurons becoming completely unresponsive to these neurotrophins with age or to a marked shift in the dose

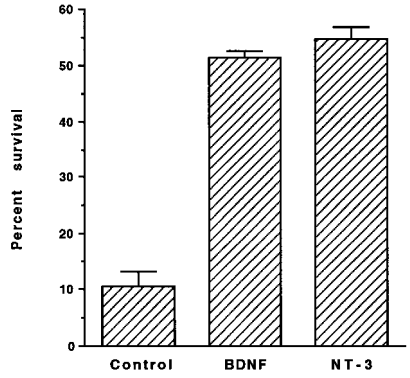

Fig. 3. BDNF and NT-3 exert a direct survival-promoting effect on E6 DMTG neurons. Bar chart of the percentage survival after 48 hours incubation of cohorts of E6 DMTG neurons identified 6 hours after plating. Cohorts were grown either without neurotrophins (control) or were exposed to 2 ng/ml BDNF or NT-3 at 6 hours. The mean ± the standard error are shown (n=3).

response of neurons in the population as a whole. To distinguish between these possibilities, E6-E12 DMTG neurons were grown with BDNF or NT-3 over a broad range of concentrations up to 5 μg/ml (which is over 4 orders of magnitude higher than the saturating concentration of these neurotrophins for nodose and VLTG neurons, data not shown). For comparison, dose responses of DMTG neurons to NGF were also carried out at these ages.

Fig. 4 shows the dose responses of E6, E8, E10 and E12 DMTG neurons to NGF, BDNF and NT-3. At E6, the majority of DMTG neurons were supported by saturating concentrations of each neurotrophin, and the concentrations of each neurotrophin that promoted half-maximal survival were similar (3.8, 4.7 and 12.7 pg/ml for NGF, BDNF and NT-3, respectively). By E8, however, the BDNF and NT-3 dose-responses showed a marked shift to higher concentrations. Although the majority of neurons were still promoted by the highest concentration of BDNF and NT-3 used (5 μg/ml), the concentrations of BDNF and NT-3 that promoted half-maximal survival had increased by 4 orders of magnitude by E8 (to 98 and 124 ng/ml for BDNF and NT-3, respectively). There appeared to be further shifts in the BDNF and NT-3 dose responses to higher concentrations at E10 and E12 (seen most clearly in the case of NT-3). However, because maximal neuronal survival was not reached at the highest concentration used (5 μg/ml), the half-maximally effective concentrations cannot be reliably calculated at these ages.

There was also a shift in the NGF dose response to higher concentrations between E6 and E8. However, in contrast to the very large shifts in the BDNF and NT-3 dose responses during this period, the half-maximally effective NGF concentration increased by only one order of magnitude from 3.8 to 28 pg/ml. In contrast to the continued shifts in the BDNF and NT-3 dose responses at later ages, the NGF dose responses were similar at E8, E10 and E12 and there was no significant difference in the half-maximally effective NGF concentrations at these ages.

DISCUSSION

In agreement with previous in vitro studies of the effects of NGF and BDNF on the survival of mid-embryonic cranial sensory neurons (Davies and Lindsay, 1985; Lindsay and Rohrer, 1985; Lindsay et al., 1985; Davies et al., 1986a,b), we have shown that NGF promotes the survival of DMTG and jugular neurons and BDNF promotes the survival of VLTG and nodose neurons grown in low-density cultures that are free of non-neuronal cells. In addition, by studying neurons at earlier developmental stages, we have shown that the survival of NGF-dependent DMTG and jugular neurons is transiently supported by BDNF and NT-3. Because there was negligible additional survival in E6 DMTG and jugular cultures grown with combinations of NGF, BDNF and NT-3, most of these neurons responded to all three factors during this early stage of their development. By monitoring the survival of individual E6 DMTG neurons between 6 and 48 hours in vitro, we showed BDNF and NT-3 exert a direct, through transient, survival-promoting action on NGF-dependent sensory neurons at an early stage in their development.

Previous work on the NGF-dependent trigeminal ganglion neurons of the mouse embryo has shown that the transient survival-promoting effects of BDNF and NT-3 on these neurons is largely over by the time the number of neurons in the trigeminal ganglion begins to decline as a result of naturally occurring neuronal death (Buchman and Davies, 1993). Although there are no available data on the timing of neuronal death in the chicken trigeminal ganglion, a developmental study of neuronal death in the sensory ganglia of cranial nerves IX and X in chicken embryos (Hiscock and Straznicky, 1986) has shown that the total number of neurons in the jugular ganglion peaks at E8 and decreases by half over the next five days of development. Thus, at least for chicken jugular ganglion neurons, the timing of the early survival response to BDNF and NT-3 appears to be similar to that observed for mouse trigeminal ganglion neurons in that it is largely over by the time neuron numbers begin to fall as a result of cell death.

In vitro studies of developing dorsal root ganglion (DRG) neurons have shown that NGF and BDNF supported the survival of distinct subsets of neurons at E12, whereas at E6 more than half of the neurons were supported by either factor alone (Lindsay et al., 1985; Acheson et al., 1987), indicating that there is some overlap between NGF-responsive and BDNF-responsive neurons during the early stages of DRG development. In a detailed in vitro study of the survival requirements of DRG neurons from the earliest stages of their development (Ernsberger and Rohrer, 1988), it was shown that after an initial phase of neurotrophin independence, the survival of all neurons was promoted by either NGF or BDNF and that a proportion of the neurons subsequently lost responsiveness to BDNF. Our current observations of early DMTG and jugular neurons are consistent with the early response of NGF-dependent neurons to BDNF and the subsequent loss of BDNF responsiveness. In addition, we show that NGF-dependent neurons are also transiently supported by NT-3 early in their development. Furthermore, by studying several different populations of cranial sensory neurons, we have been able to show that the broad neurotrophin responsiveness of NGF-dependent neurons at early developmental stages does not extend to other populations of developing sensory neurons. VLTG neurons are supported by BDNF throughout their early development, but show virtually no response to either NGF or NT-3. Nodose neurons respond to BDNF and NT-3 from an early developmental stage, but show negligible response to

NGF at any stage in their development. Thus, responsiveness to multiple neurotrophins is not a ubiquitous feature of the early stages of neuronal development, but among sensory neurons appears to be restricted to those that depend on NGF for survival during the phase of naturally occurring cell death.

We have shown that the loss of the response of the NGF-dependent DMTG neurons to BDNF and NT-3 during development is due to marked shifts in the BDNF and NT-3 dose responses to higher concentrations. Between E6 and E8 there is a shift of over four orders of magnitude in the BDNF and NT-3 dose responses and further increases are apparent at later stages. Two kinds of transmembrane glycoproteins have been shown to be receptors for neurotrophins: p75 and members of the trk family of receptor tyrosine kinases (Chao, 1992; Meakin and Shooter, 1992). Whereas p75 binds all neurotrophins with similar low affinity (Sutter et al., 1979; Rodriguez-Tébar and Barde, 1990; Hallbook et al., 1991; Rodriguez-Tebar et al., 1992), trk tyrosine kinases are bound and undergo transphosphorylation selectively by neurotrophins. Studies in cell lines suggest that NGF is the preferred ligand for TrkA (Hempstead et al., 1991; Kaplan et al., 1991a,b; Klein et al., 1991a), BDNF for trkB (Glass et al., 1991; Klein et al., 1991b, 1992; Soppet et al., 1991; Squinto et al., 1991) and NT-3 for trkC (Lamballe et al., 1991). The marked shifts in the BDNF and NT-3 responses may be due to reduced levels of expression of trkB and trkC in DMTG neurons with development, or to the increasing expression of truncated trkB and trkC isoforms that may act as dominant-negative receptors (Jing et al., 1992). Interestingly, there is a smaller shift of one order of magnitude in the NGF dose response of DMTG neurons to higher NGF concentrations between E6 and E8. A similar shift in the NGF dose response is observed in mouse trigeminal neurons during a roughly equivalent stage of development between E12 and E15 (Buchman and Davies, 1993). Curiously, the mean level of *trkA* mRNA increases in trigeminal neurons over this period of development (Wyatt and Davies, 1993) and there is no evidence for the existence of a trkA isoform lacking the tyrosine kinase domain in developing trigeminal neurons (Rosenthal and Davies, unpublished findings). This developmental shift in the NGF dose response is also not dependent on the expression of p75 because it is still observed in developing trigeminal neurons obtained from mouse embryos that have a null mutation of the p75 gene (Davies et al., 1993). When chicken-specific probes for trks and their isoforms become available, embryonic chicken DMTG neurons will undoubtedly be a useful model system for studying the molecular basis of selective developmental shifts in neurotrophin sensitivity.

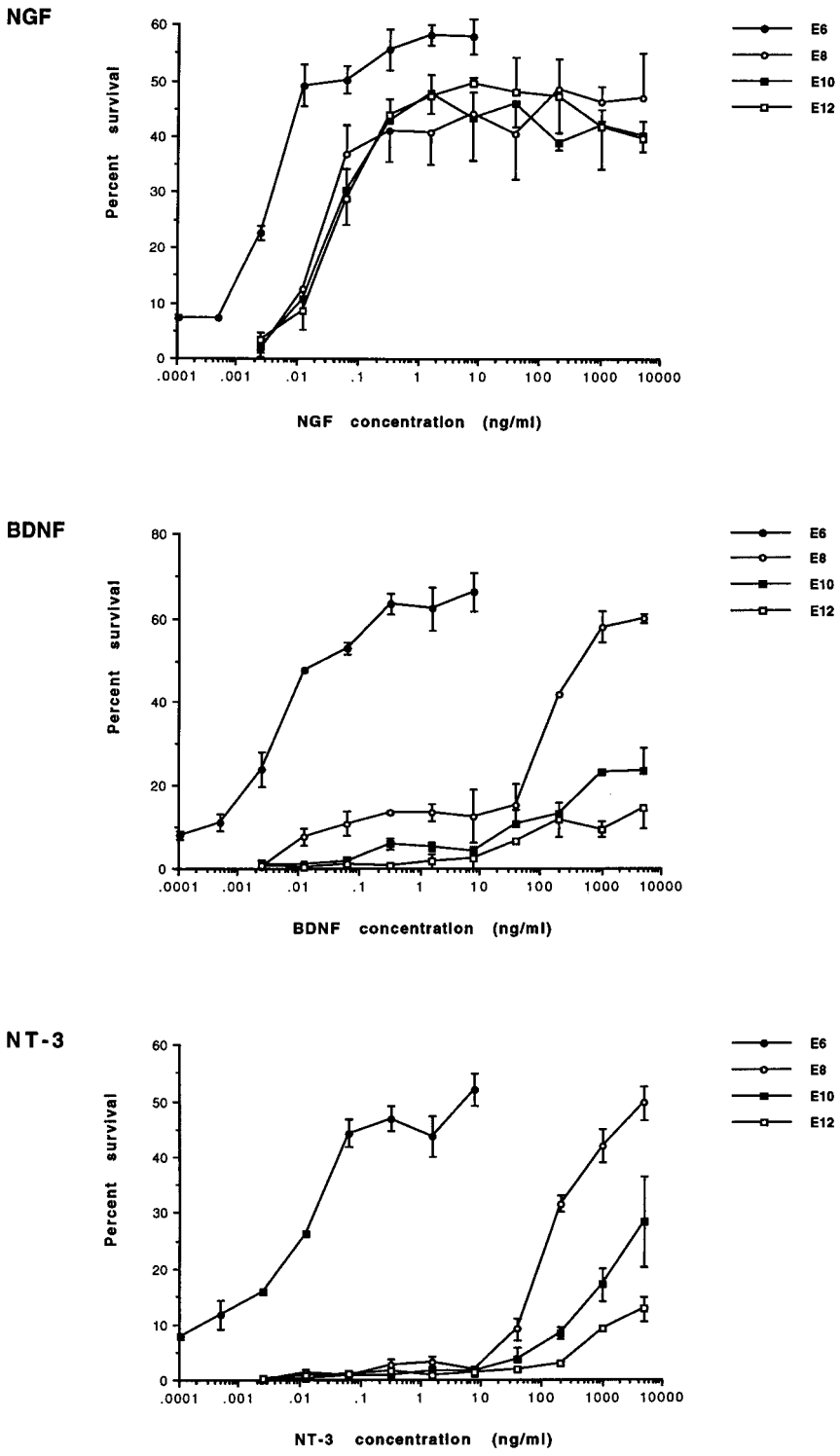

Fig. 4. Dose responses of DMTG neurons to neurotrophins. Graphs showing the percentage survival of DMTG neurons from E6 (filled circles), E8 (open circles), E10 (filled squares) and E12 (open squares) embryos after 48 hours incubation with NGF, BDNF or NT-3 at concentrations ranging from 0.1 pg/ml to 5 μg/ml. The mean ± the standard error from typical experiments are shown.

This work was supported by grants from Action Research and the Wellcome Trust. We thank Gene Burton and John Winslow of Genentech Inc. for the purified recombinant NGF, BDNF and NT-3.

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(Accepted 10 February 1994)