Developmental changes in the response of trigeminal neurons to neurotrophins: influence of birthdate and the ganglion environment

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SUMMARY

Previous studies have shown that most neurons in cultures established during the early stages of neurogenesis in the embryonic mouse trigeminal ganglion are supported by BDNF whereas most neurons cultured from older ganglia survive with NGF. To ascertain to what extent these developmental changes in neurotrophin responsiveness result from separate phases of generation of BDNF- and NGF-responsive neurons or from a developmental switch in the response of neurons from BDNF to NGF, we administered BrdU to pregnant mice at different stages of gestation to identify neurons born at different times and studied the survival of labelled neurons in dissociated cultures established shortly after BrdU administration. Most early-generated neurons responded to BDNF, neurons generated at intermediate times responded to both factors and late-generated neurons responded to NGF, indicating that there are overlapping phases in the generation of BDNF- and NGF-responsive neurons and that late-generated neurons do not switch responsiveness from BDNF to NGF. To ascertain if early-generated neurons do switch their response to neurotrophins during development, we used repeated BrdU injection to label all neurons generated after an early stage in neurogenesis and

studied the neurotrophin responsiveness of the unlabelled neurons in cultures established after neurogenesis had ceased. The response of these early-generated neurons had decreased to BDNF and increased to NGF, indicating that at least a proportion of early-generated neurons switch responsiveness to neurotrophins in vivo. Because early-generated neurons do not switch responsiveness from BDNF to NGF in long-term dissociated cultures, we cultured early trigeminal ganglion explants with and without their targets for 24 hours before establishing dissociated cultures. This period of explant culture was sufficient to enable many early-generated neurons to switch their response from BDNF to NGF and this switch occurred irrespective of presence of target tissue. Our findings conclusively demonstrate for the first time that individual neurons switch their neurotrophin requirements during development and that this switch depends on cell interactions within the ganglion. In addition, we show that there are overlapping phases in the generation of BDNF- and NGF-responsive neurons in the trigeminal ganglion.

Key words: Neuron, Mouse, Trigeminal, BDNF, NGF

INTRODUCTION

The neurotrophins (NGF, BDNF, NT-3 and NT-4) are a family of structurally related secreted proteins that promote the survival of a variety of neurons in the developing vertebrate nervous system by signalling via members of the Trk family of receptor tyrosine kinases (Lewin and Barde, 1996; Davies, 1997). TrkA is a receptor for NGF, TrkB is a receptor for BDNF and NT4 and TrkC is a receptor for NT-3, although NT-3 can also signal less efficiently via TrkA and TrkB in some neurons (Klein, 1994; Bothwell, 1995). Whereas some populations of neurons are dependent on only one neurotrophin for survival during development, the survival of many other populations is regulated by several different neurotrophins. Some populations of neurons may require several different neurotrophins during a particular stage of their development

whereas other populations may switch their neurotrophin survival requirements during development (Davies, 1997).

One of the best characterised examples of a population of neurons whose survival requirements switch from one set of neurotrophins to another during development are those of the embryonic mouse trigeminal ganglion. In cultures established from E10 embryos, the majority of neurons survive with either BDNF or NT-3, and very few respond to NGF. In cultures established at later stages, NGF supports an increasing proportion of the neurons, BDNF and NT-3 support progressively fewer neurons and, by E13, most neurons survive with NGF but few survive with BDNF or NT-3. In E11 and E12 cultures, there is negligible additional neuronal survival in cultures containing NGF plus BDNF or NT-3, suggesting that, at this stage, most neurons are capable of responding to all three neurotrophins (Buchman and Davies, 1993; Paul and

Davies, 1995). These changes in neurotrophin responsiveness in vitro are mirrored by changes in the expression of Trk receptors in vivo. At early developmental stages, TrkB and TrkC mRNA are highly expressed and TrkA mRNA is poorly expressed. With age, TrkB and TrkC expression becomes restricted to a small number of neurons, there is a relative increase in truncated TrkB, which acts as a negative modulator of BDNF signalling, and TrkA mRNA is expressed by increasing numbers of neurons (Ernfors et al., 1992; Arumae et al., 1993; Wyatt and Davies, 1993; Ninkina et al., 1996). Accordingly, the number of trigeminal neurons undergoing apoptosis is increased during early developmental stages in $trkB^{-/-}$ embryos and is increased later in development in $trkA^{-/-}$ embryos (Piñón et al., 1996). Thus, many neurons depend on TrkB signalling for survival in the early trigeminal ganglion before becoming dependent on TrkA signalling. Although early trigeminal neurons survive in culture equally well with either of the two preferred TrkB ligands, BDNF and NT-4 (Davies et al., 1993a), BDNF appears to be the physiologically relevant TrkB ligand for these neurons in vivo because there is a significant reduction in the neuronal complement of the neonatal trigeminal ganglia of $BDNF^{-/-}$ mice (Ernfors et al., 1994; Jones et al., 1994) but not $NT-4^{-/-}$ mice (Conover et al., 1995; Liu et al., 1995). There is also a large decrease in the neuronal complement of the trigeminal ganglion of $NT-3^{-/-}$ embryos before the peak of naturally occurring neuronal death (ElShamy and Ernfors, 1996; Wilkinson et al., 1996), implying that, like BDNF, endogenous NT-3 is also required at an early stage in trigeminal ganglion development. However, because apoptosis is not increased to the same extent in early $trkC^{-/-}$ embryos (Piñón et al., 1996; Tessarollo et al., 1997), it is possible that NT-3 acts via TrkA or TrkB in vivo as has been demonstrated in TrkC-deficient trigeminal neurons in vitro (Davies et al., 1995).

Taken together, the above findings have led to the view that many neurons generated during the early phase of neurogenesis in the trigeminal ganglion switch their survival requirements from BDNF or NT-3 to NGF as they develop (Davies, 1997). Furthermore, the finding that E10 trigeminal neurons survive with BDNF for at least 5 days in vitro without acquiring responsiveness to NGF and that increasing numbers of neurons in E11 and E12 cultures survive after being switched from BDNF to NGF has led to the idea that the proposed switch from BDNF to NGF dependence is due to some signal that acts on the neurons in vivo (Paul and Davies, 1995). However, because neurons are generated in the trigeminal ganglion from E9.5 to E13 (Davies and Lumsden, 1984; Wilkinson et al., 1996) and because the majority of neurons respond to NGF by E12 (Buchman and Davies, 1993), it is possible that late-born neurons do not pass through a transient period of responsiveness to BDNF and NT-3 before becoming NGF responsive, but respond to NGF from the outset.

To determine if the initial neurotrophin response of trigeminal neurons is related to birthdate and to ascertain whether or not early-born neurons really do switch neurotrophin dependence in vivo, we have used BrdU incorporation in utero to identify trigeminal neurons born at different stages of neurogenesis and analysed the response of these neurons to different neurotrophins in vitro. We have restricted the analysis to BDNF and NGF because trigeminal neurons exhibit clearly saturable dose responses to these

factors and because they signal by different Trk receptors, TrkB and TrkA, respectively (Buchman and Davies, 1993). In contrast, the developmental significance of the effects of NT-3 on neuronal survival in vitro is difficult to interpret because it acts on all Trk receptors with different efficiencies which change during trigeminal development (Davies et al., 1995). As a consequence, trigeminal neurons lack a clear sigmoidal dose response to NT-3 and almost all trigeminal neurons can be supported by NT-3 in vitro if sufficiently high concentrations are used (Buchman and Davies, 1993; Davies et al., 1995). Thus, although NT-3 plays a role in promoting the survival of a subset of trigeminal neurons in vivo (ElShamy and Ernfors, 1996; Wilkinson et al., 1996), the survival response of trigeminal neurons to NT-3 observed in culture neither clearly indicates the kinds of Trk receptors expressed nor gives a clear indication of the in vivo role of NT-3.

Our present findings show that many early-born neurons do indeed switch neurotrophin responsiveness during development and demonstrate that late-born neurons respond to NGF shortly after becoming postmitotic without going through a transient period of responsiveness to BDNF. Furthermore, explant studies indicate that a signal acting within the cellular environment of the early trigeminal ganglion is sufficient to enable early-born trigeminal neurons to switch neurotrophin dependence.

MATERIALS AND METHODS

Dissociated neuron cultures

Trigeminal ganglia were dissected from CD1 mouse embryos at different stages of gestation and were trypsinised and dissociated into a cell suspension that was seeded in poly-ornithine/laminin-coated culture dishes in defined medium as described previously (Davies et al., 1993b). For neuron survival studies not involving BrdU labelling, the cells were plated in 35 mm tissue culture Petri dishes (Nunc) at a density of 500 to 1,000 per dish. 6-12 hours after plating, the number of attached neurons within a 12×12 mm grid in the centre of each dish was counted; the mean of these counts was taken as the initial number of neurons for the experiment. After 48 hours incubation, the percentage neuronal survival in control cultures (no added neurotrophins) or cultures supplemented with BDNF, NGF or BDNF plus NGF was estimated by counting the number of surviving neurons in the grid and expressing the results as a percentage of the initial number of attached neurons. The small percentage of neurons surviving in control cultures in each experiment (<10%) was subtracted from per cent survival in the neurotrophin-supplemented cultures. The neurotrophins were used at a concentration of 10 ng/ml which is well above saturation in the dose response of developing trigeminal neurons to these factors (Buchman and Davies, 1993). In each experiment, triplicate cultures were set up for all conditions. Each type of experiment was carried out a minimum of three times.

Explant cultures

Explants consisted of trigeminal ganglia alone or single trigeminal ganglia with the adjoining peripheral and/or central target tissues still attached. The peripheral target included in these explants was the maxillary process, the most densely innervated part of the peripheral target field (Davies and Lumsden, 1984). The central target included in these explants was the adjoining part of the metencephalon, which contains the principal trigeminal sensory nucleus. The explants were incubated in defined medium with or without neurotrophins in bacteriology dishes (so that they did not adhere to the substratum).

BrdU labelling

Pregnant mice were injected intraperitoneally at particular stages of gestation with BrdU at concentrations ranging from 5 to 50 mg per kg body weight (see Results for details of each set of experiments). Dissociated cultures established from the ganglia were plated in poly-ornithine/laminin-coated 48-well plates in defined medium. The cultures were fixed for 20 to 30 minutes with 4% paraformaldehyde, washed with PBS, permeablised with 0.5% Tween in PBS, treated with 0.3 M HCl for 1.5 hours to denature the DNA and incubated with 1:400 anti-BrdU monoclonal antibody (Sigma) overnight at 4°C. After washing with PBS, the cultures were incubated with biotinylated goat anti-rabbit antiserum diluted 1:500 in PBS for 3 hours followed by further rinses with PBS and incubation for 30 minutes with an avidin/biotinylated horseradish peroxidase macromolecular complex (Vector Laboratories). After rinsing with PBS, peroxidase activity was localised by incubation with a 0.5 mg/ml diaminobenzidine solution in PBS containing 0.01% hydrogen peroxide, 0.01% nickel chloride and 0.01% cobalt chloride. In each experiment, cultures were processed for BrdU incorporation between 6 and 12 hours after plating to determine the initial proportion of BrdU-positive and BrdU-negative neurons in the cultures and after a further 36 hours incubation with and without neurotrophins. Triplicate wells were set up for each condition in every experiment, and between 200 and 500 neurons were scored as BrdU-positive or BrdU-negative in each well. Each type of experiment was carried out a minimum of three times.

Measurement of TrkA mRNA levels

A quantitative RT-PCR technique was used to measure the levels of TrkA mRNA in total RNA extracted from explant and dissociated cultures of trigeminal ganglia (Wyatt and Davies, 1993). The reverse transcription and PCR reactions were calibrated by the inclusion of known amounts of a specific TrkA cRNA competitor template in the reverse transcription reaction. To compare the levels of TrkA mRNA in different experiments, we also used quantitative RT/PCR to measure the level of the mRNA that encodes the housekeeping protein glyceraldehyde phosphate dehyrodenase (GAPDH), and expressed the level of TrkA mRNA relative to GAPDH mRNA. Details of the competitors, primers and reaction conditions are provided elsewhere (Wyatt and Davies, 1993). Previous detailed comparisons of this method with quantitative northern blotting has demonstrated its accuracy and reproducibility over a wide range of mRNA concentrations (Wyatt and Davies, 1993; Wyatt et al., 1997).

RESULTS

Neurons born at different stages differ in their response to neurotrophins

To determine if the responsiveness of trigeminal neurons to neurotrophins differs with birth date, pregnant mice were injected with BrdU at different stages throughout the period of neurogenesis in the trigeminal ganglion. The mice were killed 5 hours later and dissociated cultures of trigeminal neurons were established from the embryos. These cultures were fixed and stained for nuclear incorporation of BrdU shortly after plating to determine the initial number of BrdU-positive and BrdU-negative neurons in these cultures, and after 48 hours incubation in parallel cultures with BDNF, NGF or BDNF plus NGF to determine the numbers of BrdU-positive and BrdU-negative neurons that survive with these factors (Fig. 1A).

In accordance with the known period of neurogenesis in the trigeminal ganglion from E9.5 to E13 (Davies and Lumsden, 1984; Wilkinson et al., 1996), the percentages of BrdU-positive neurons shortly after plating in cultures established from embryos treated with BrdU for 5 hours in utero decreased markedly from E10 to E12.5 (Fig. 1B). Fig. 1C shows that virtually all BrdU-positive neurons in cultures established from embryos treated with BrdU at E10 survived for 48 hours in medium containing BDNF whereas very few of these neurons survived with NGF. This indicates that neurons born shortly after E10 initially survive in response to BDNF but not NGF. The majority of BrdU-positive neurons in cultures established from embryos treated with BrdU at E10.5 survived in response to either BDNF or NGF and the number of surviving BrdU-positive neurons was not significantly different in cultures containing BDNF plus NGF. This indicates that neurons born shortly after E10.5 respond equally well to BDNF and NGF. The great majority of BrdU-positive neurons in cultures established from embryos treated with BrdU at E11.5 survived in response to NGF and very few survived with BDNF. This indicates that the great majority of neurons born shortly after E11.5 survive in response to NGF not BDNF, demonstrating that late-born neurons in the trigeminal ganglion do not switch responsiveness from BDNF to NGF but start responding to NGF from the outset.

Early-born neurons change responsiveness to neurotrophins during development

Although early-born trigeminal neurons initially respond to BDNF, because cultures established from older ganglia contain very few BDNF-responsive neurons, it is possible that some of the early-born neurons switch responsiveness from BDNF to NGF in vivo. To test this directly, we needed a method of identifying early-born neurons in cultures that were established from older trigeminal ganglia. We did this by making repeated injections of BrdU into pregnant mice at daily intervals from an early stage in development so that all neurons born after injections commenced would be labelled by BrdU whereas those born before this time would be unlabelled. We first carried out a series of experiments to establish a labelling protocol that would reliably label all neurons in the ganglion if commenced at the onset of neurogenesis. We found that BrdU administered at daily intervals from E9.5 was sufficient to achieve this. Virtually all of the neurons were labelled by this protocol in cultures established at E12.5; repeated doses of 5, 10 or 20 mg BrdU per kg body weight being equally effective (99.43±0.03, 99.63±0.11 and 99.64±0.08% labelled neurons, respectively). Moreover, the relative proportions of neurons surviving with BDNF, NGF or BDNF plus NGF in cultures established from animals treated with BrdU at each of these concentrations were virtually identical to those in cultures established from untreated animals (data not shown). These results show that repeated BrdU administration throughout the period of neurogenesis successfully labels virtually all neurons but does not affect the characteristic survival response of the neurons to neurotrophins at the end of the period of neurogenesis.

Having established a method of identifying early-born neurons, we decided to compare the response of neurons born before E10.5 in cultures established shortly after E10.5 (when almost all neurons respond to BDNF) and cultures established at E13.5 (after neurogenesis had ceased in the ganglion when almost all neurons respond to NGF). Fig. 2 shows that almost all unlabelled neurons in cultures established 5 hours after administering BrdU to E10.5 embryos survive in response to

BDNF and very few survive with NGF, demonstrating that almost all neurons born before E10.5 are initially dependent on BDNF for survival. However, unlabelled neurons in cultures established from E13.5 embryos that had been exposed to repeated doses of BrdU administered at E10.5, E11.5 and E12.5 (i.e., neurons born before E10.5) showed a greatly increased response to NGF and a markedly reduced response to BDNF, suggesting that many early-born neurons had gained responsiveness to NGF during the ensuing 3 days in vivo whereas most had lost responsiveness to BDNF. Interestingly, NGF and BDNF had an additive effect on the survival of early-born neurons in cultures established from these older ganglia (Fig. 2B), suggesting that neurons born before E10.5 have segregated into a separate minor subpopulation of BDNF-dependent neurons and a separate major population of NGF-dependent neurons by E13.5.

Early-born neurons change responsiveness to neurotrophins in explant cultures

It has been shown that cultures of neurons established from early trigeminal ganglia do not switch responsiveness from BDNF to NGF in culture but retain dependence on BDNF for at least 5 days in vitro (Paul and Davies, 1995). However, in the present study, we have demonstrated that these same neurons change their neurotrophin dependence with age in vivo, suggesting that the neurons are exposed to a signal in vivo that promotes this switch. It is conceivable that this signal originates from either the peripheral or central target fields of the neurons or from within the ganglion itself. To explore these possibilities, we grew E10.5 trigeminal ganglion explants alone or with their peripheral or central target tissues attached for 24 hours before dissociating the ganglia and establishing low-density cultures in medium containing NGF or BDNF. Whereas the majority of neurons in dissociated cultures of E10.5 neurons established directly after dissection survived in response to BDNF but not NGF after both 48 and 72 hours in vitro, the majority of neurons in cultures of E10.5 neurons that were grown first as explants for 24 hours prior to dissociation survived with NGF not BDNF (Fig. 3). This difference in response to neurotrophins is very similar to that observed in dissociated cultures of E11.5 neurons after 48 hours incubation (i.e., at the equivalent stage of development to E10.5 neurons grown in dissociated culture for 72 hours). The

same results were observed irrespective of the inclusion of the peripheral or central target fields in the E10.5 explant (Fig. 3). The same results were also obtained whether or not neurotrophins were present during the 24 hour explantation period (data not shown). These results suggest that a signal within the ganglion plays a role in switching the response of some early trigeminal neurons from BDNF to NGF.

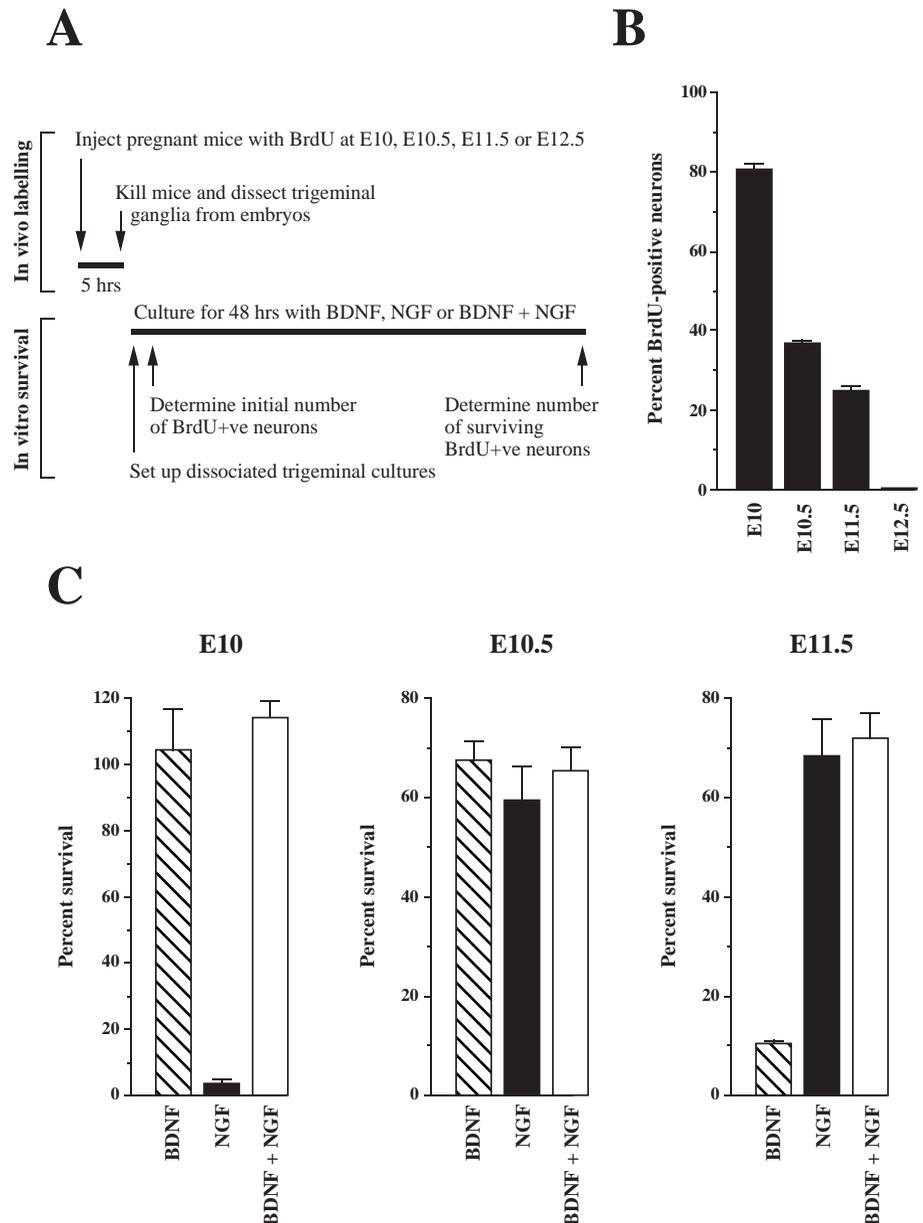Because many neurons generated in the trigeminal

Fig. 1. Trigeminal neurons born at different stages of development differ in their response to neurotrophins. (A) Illustration of the experimental paradigm from left to right, beginning with in vivo BrdU incorporation followed by assessment of the in vitro survival of labelled neurons in dissociated cultures supplemented with neurotrophins. (B) Bar chart of the initial numbers of BrdU-positive neurons in these cultures shortly after plating expressed as a percentage of total number of neurons (BrdU-positive plus BrdU-negative). (C) Bar charts showing the neurotrophin survival responses of BrdU-positive neurons after 48 hours incubation in dissociated cultures established from embryos that were exposed to BrdU in utero at 10, 10.5 and 11.5 days gestation. The mean and s.e.m. of the number of BrdU-positive neurons surviving with BDNF, NGF or BDNF plus NGF is shown as a percentage of the number of BrdU-positive neurons estimated shortly after plating (n=3).

ganglion between E10.5 and E11.5 in vivo respond to NGF from the outset (Fig. 1C), it is possible that neurons generated in E10.5 trigeminal ganglia grown as explants for 24 hours could account, at least in part, for the above results. To address this issue, we grew E10.5 explants in the presence of BrdU for 24 hours prior to the establishment of dissociated cultures to distinguish between neurons that were born in the ganglia prior to and after explantation (Fig. 4A). In dissociated cultures established from these explants, about half of the neurons shortly after plating (51.6±0.8%) were labelled with BrdU, indicating that a substantial number of neurons had been generated in the ganglion explants in vitro. As expected, the great majority (72%) of these recently generated neurons was supported by NGF in dissociated cultures established from the explants and few survived with BDNF (Fig. 4B). However, many of the unlabelled neurons (i.e., those born prior to explantation) had also become more responsive to NGF and less responsive to BDNF (Fig. 4B) compared with E10.5 neurons that were grown in dissociated cultures from the outset (Fig. 3). This suggests that many neurons born before E10.5 had indeed switched their responsiveness from BDNF to NGF within the ganglion explant.

NGF responsiveness in explant culture is correlated with increased trkA mRNA expression

NGF promotes the survival of neurons by binding to the TrkA receptor tyrosine kinase (Klein et al., 1991; Kaplan et al., 1991; Allsopp et al., 1993; Xu et al., 1994). Previous developmental studies have shown that the appearance of NGF-responsive neurons in the trigeminal ganglion in vivo is associated with a marked increase in the expression of TrkA mRNA (Wyatt and Davies, 1993). To determine if the appearance of NGF-responsive neurons in early trigeminal neurons grown in explants, as opposed to those grown in dissociated culture, is

Fig. 2. Early-born neurons change responsiveness to neurotrophins during development. (A) Illustration of the experimental paradigm from left to right, beginning with in vivo BrdU incorporation commencing at 10.5 days gestation followed by assessment of the in vitro survival of unlabelled neurons (i.e., those born before E10.5) in dissociated cultures set up either 5 hours after BrdU administration (upper flow diagram) or after a further 3 days of repeated BrdU administration (lower flow diagram). (B) Bar charts showing the neurotrophin survival responses of BrdU-negative neurons after 48 hours incubation in dissociated cultures established from embryos that were exposed to BrdU in utero for either 5 hours at E10.5 (first bar chart) or for 3 days from E10.5 to E13.5 (second bar chart). In both bar charts, the mean and s.e.m. of the number of BrdU-negative neurons surviving with BDNF, NGF or BDNF plus NGF is shown as a percentage of the number of BrdU-negative neurons estimated shortly after plating ($n=3$).
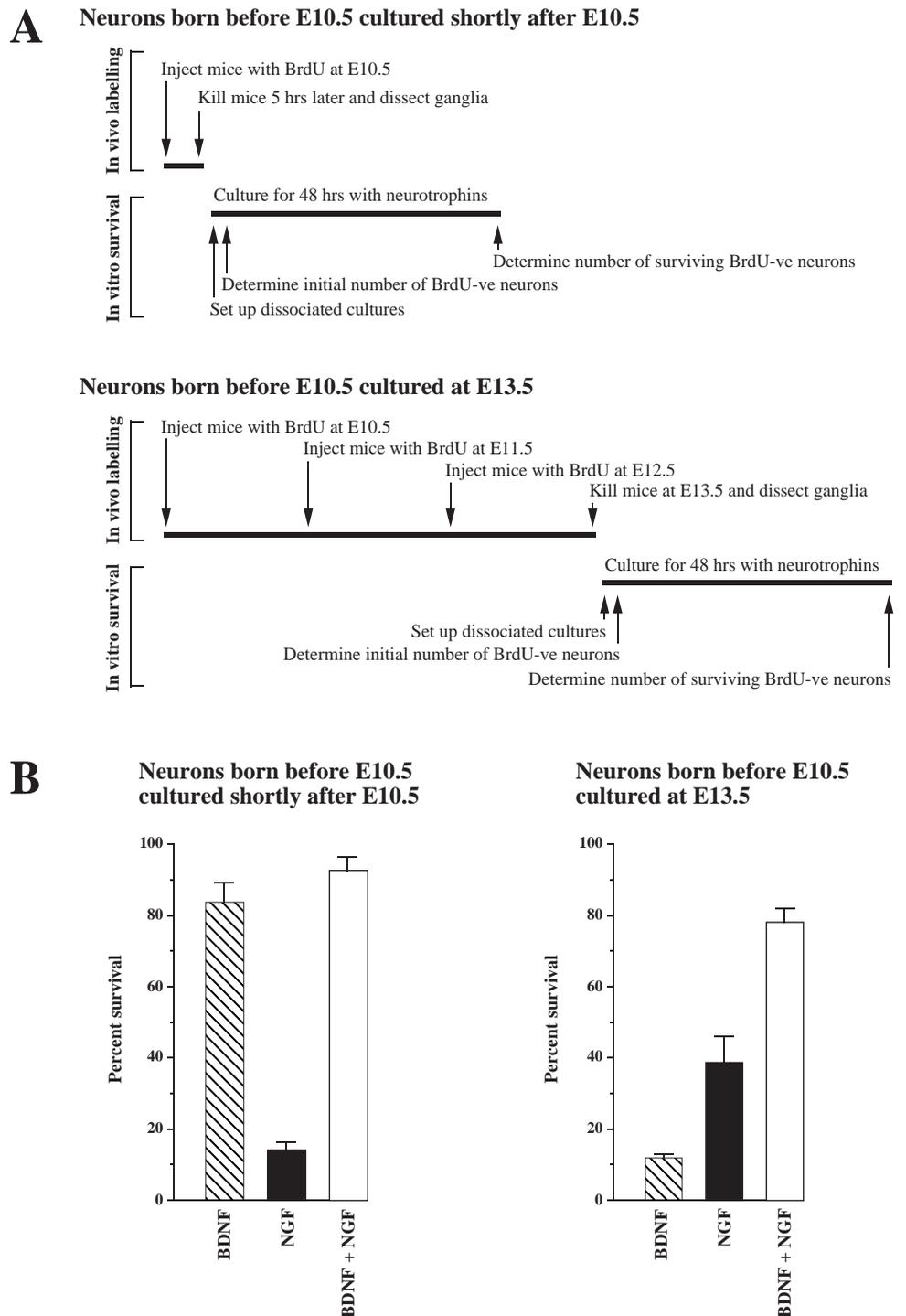
correlated with upregulation of TrkA mRNA expression, we used quantitative RT/PCR (Wyatt and Davies, 1993) to measure the levels of TrkA mRNA in E10.5 explants and dissociated cultures. Fig. 5 shows that there was a marked increase in the level of TrkA mRNA in trigeminal explants after 24 hours incubation whereas the level of TrkA mRNA was not sustained in low-density dissociated cultures. Although the majority of neurons in E10.5 low-density dissociated cultures survive without neurotrophins for 24 hours (Buchman and Davies, 1993), to exclude the possibility that the low level of TrkA mRNA expression in these cultures was due to suboptimal neuronal survival, TrkA mRNA was also measured in cultures supplemented with NGF and BDNF. The level of TrkA mRNA in these neurotrophin-supplemented dissociated cultures was similar to that in control cultures (Fig. 5). Likewise, the presence of neurotrophins in explant cultures had little effect on TrkA mRNA expression; in both these cultures and control cultures, the level of TrkA mRNA increased markedly after 24 hours incubation (Fig. 5). Because previous studies have shown that TrkA mRNA expression is restricted to neurons in the developing trigeminal ganglion (Wyatt and Davies, 1993), these results suggest that the appearance of NGF-responsive neurons in early trigeminal explants is due to the expression of TrkA receptors on a subset of neurons in these explants whereas the inability of E10.5 neurons to acquire NGF responsiveness in dissociated culture is due to failure of the normal developmental increase in TrkA expression to occur in these neurons.

DISCUSSION

Our previous work on the development of the embryonic mouse trigeminal ganglion has shown that most neurons in cultures established early during the period of neurogenesis survive with either BDNF or NT-3 in the culture medium whereas most neurons in cultures established later in development survive with NGF (Buchman and Davies, 1993; Paul and Davies, 1995). These in vitro observations together with our demonstration that neuronal death is elevated in the trigeminal ganglia of $trkB^{-/-}$ embryos during the early stages of neurogenesis whereas neuronal death is elevated in the trigeminal ganglia of $trkA^{-/-}$ embryos during the later stages of neurogenesis (Piñón et al., 1996) suggested that trigeminal neurons respond to different neurotrophins at different stages of their development. However, it was not clear to what extent this switch in responsiveness within the trigeminal population as a whole is due to a switch in the response of individual neurons from BDNF/NT-3 to NGF or to different phases in the generation of BDNF/NT-3-dependent and NGF-dependent neurons.

To address this key question, we used BrdU incorporation in utero to label neurons born at different stages of development and subsequently tested the response of these neurons to neurotrophins in vitro. We focused on responses to BDNF and NGF because these are mediated by different receptors (TrkB and TrkA,

respectively) in a clear dose-dependent manner that is saturable at low concentrations (Buchman and Davies, 1993). We did not examine NT-3 responses because in vivo and in vitro studies of trigeminal neurons that lack one or more functional Trk receptors have shown that NT-3 promotes neuronal survival by signalling predominantly via TrkA and TrkB (Davies et al., 1995; Piñón et al., 1996). Our results show that most early-born neurons initially respond to BDNF whereas most neurons born in the latter half of the period of neurogenesis respond to NGF from the outset. This suggests that there are overlapping phases in the generation of neurons that respond initially to BDNF or NGF and that most late-born neurons do not switch responsiveness from BDNF to NGF. Interestingly, neurons generated at intermediate stages survive equally well with
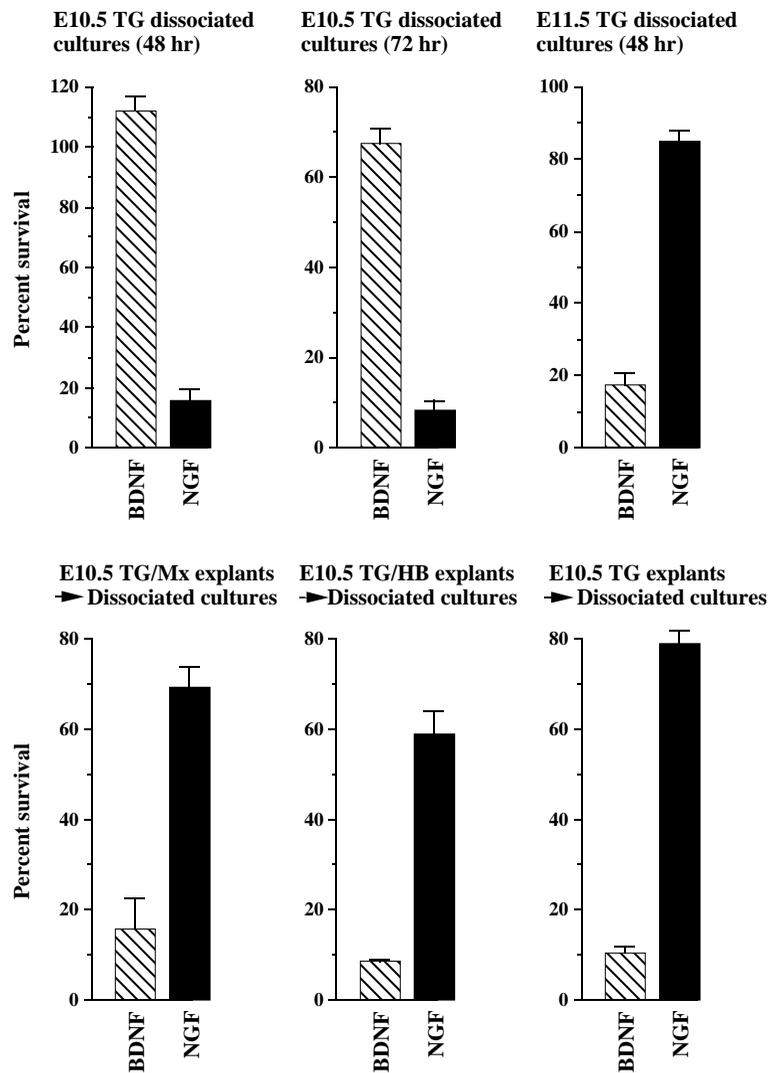

Fig. 3. Bar charts showing the survival responses of cultured trigeminal ganglion neurons to BDNF and NGF. The upper row of bar charts shows the number of E10.5 or E11.5 neurons surviving for 48 or 72 hours in dissociated cultures set up immediately after the ganglia were dissected from the embryos. The lower row of bar charts shows the number of neurons surviving in dissociated cultures of E10.5 trigeminal ganglia that were first grown as explants for 24 neurons either in combination with the maxillary process (TG/Mx), hindbrain (TG/HB) or alone (TG). The number of surviving neurons is expressed as a percentage of the number of neurons counted shortly after the dissociated neurons were plated (n ranges from 3 to 15).

either BDNF or NGF and there is no additional survival in cultures containing both factors. This suggests that these neurons are capable of being supported initially by either BDNF or NGF, implying that they co-express functionally significant levels of TrkB and TrkA at this stage in development.

Since originally submitting our paper, it has been reported in a BrdU co-labelling study that the majority of TrkB- and TrkC-immunoreactive neurons in the trigeminal ganglion are born during an early phase of neurogenesis whereas the majority of TrkA-immunoreactive neurons are born later in development (Huang et al., 1999). However, in contrast to our findings, which suggest that neurons born at intermediate stages co-express functionally significant levels of TrkB and TrkA (since they respond equally well to BDNF and NGF and there is no additional survival in the presence of both factors), Huang et al. (1999) observed only a small percentage of neurons that were immunoreactive for multiple Trk receptors in the developing trigeminal ganglion. A plausible explanation for this discrepancy between the two studies is that, whereas immunohistochemistry can unambiguously detect high levels of Trk receptor expression, this technique may not sufficiently sensitive to detect lower, yet functionally significant levels of Trk receptors. It should be noted that it has previously been shown by single-cell RT/PCR that many neurons in the developing trigeminal ganglia of rat embryos co-express mRNAs for two or all three Trk receptors (Moshnyakov et al., 1996). Furthermore, the specific anti-Trk antibodies used by Huang and colleagues recognised epitopes on the extracellular domains of these receptors and could therefore not distinguish between receptor variants that possess the intracellular tyrosine kinase domain (TK+ receptors), which is required for the survival response of neurons to neurotrophins, and receptors that lack this domain (TK− receptors). Transcripts encoding TK+ and TK− TrkB and TrkC variants are detectable in the trigeminal ganglion throughout its development from as early as E10 (Ninkina et al., 1996; Wyatt et al., 1999). The proportion of neurons co-expressing functional Trk receptors could also have been underestimated by immunohistochemistry if TK− variants are preferentially expressed in neurons containing only a single Trk receptor in the early ganglion.

The finding that TrkB and TrkC immunoreactive neurons are born before

most TrkA immunoreactive neurons in the trigeminal ganglion led to the conclusion that different waves of neurogenesis explain the developmental change in neurotrophin dependence observed in culture (Huang et al., 1999). However, neither these immunohistochemical studies nor our current demonstration that BDNF-responsive and NGF-responsive neurons are born during successive, overlapping stages of development preclude the possibility that some of the early-born neurons that initially respond to BDNF subsequently lose responsiveness to this neurotrophin and become dependent on NGF for survival. To
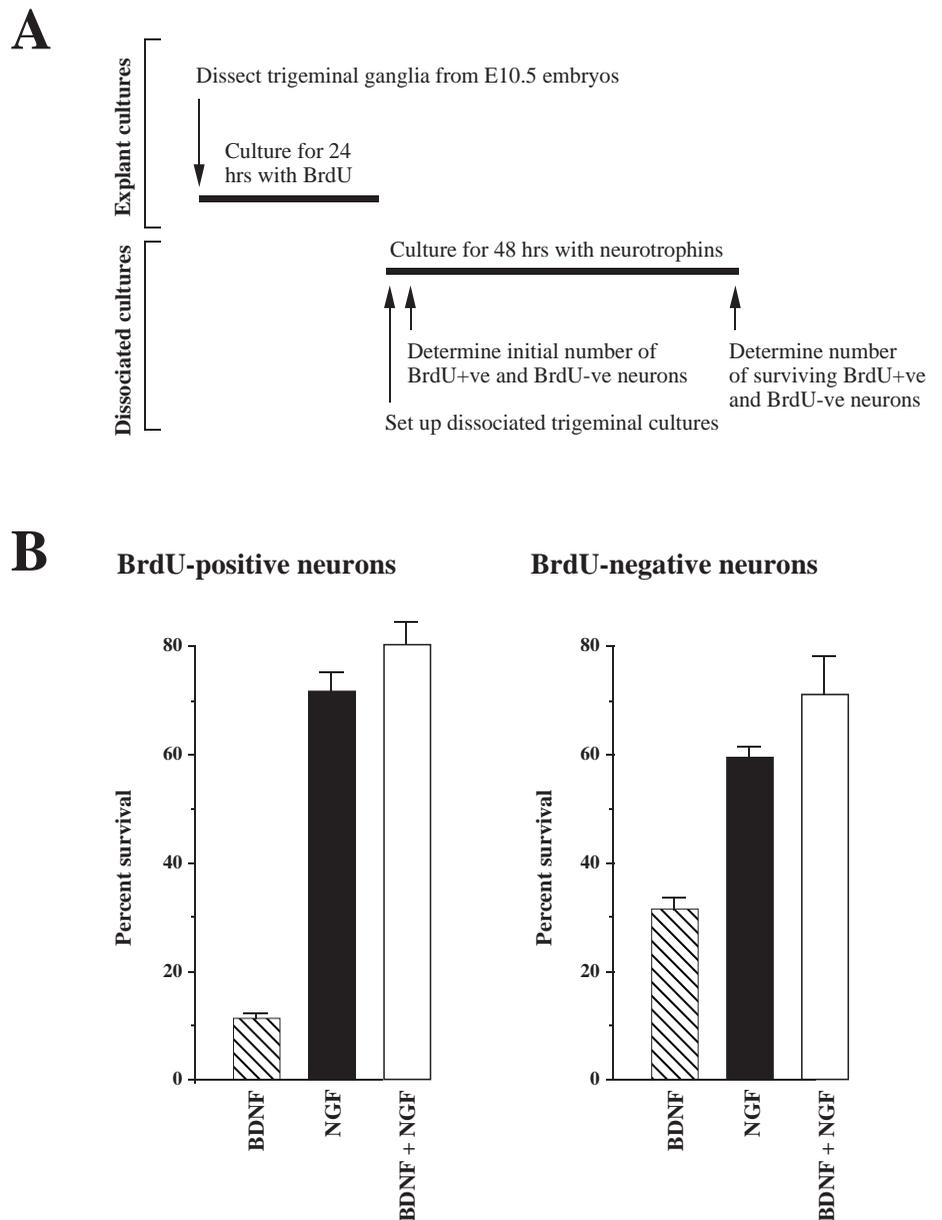

Fig. 4. Early-born neurons change responsiveness to neurotrophins in explant cultures. (A) Illustration of the experimental paradigm from left to right, beginning with BrdU incorporation in explant culture followed by assessment of the in vitro survival of labelled neurons in dissociated cultures supplemented with neurotrophins. (B) Bar charts showing the neurotrophin survival responses of BrdU-positive and BrdU-negative neurons after 48 hours in dissociated cultures of E10.5 trigeminal ganglia that were initially grown as explants for 24 hours in the presence of BrdU. The mean and s.e.m. of the final number of BrdU-positive and BrdU-negative neurons surviving with BDNF, NGF or BDNF plus NGF is shown as a percentage of the initial number of BrdU-positive and BrdU-negative neurons, respectively (n=3).
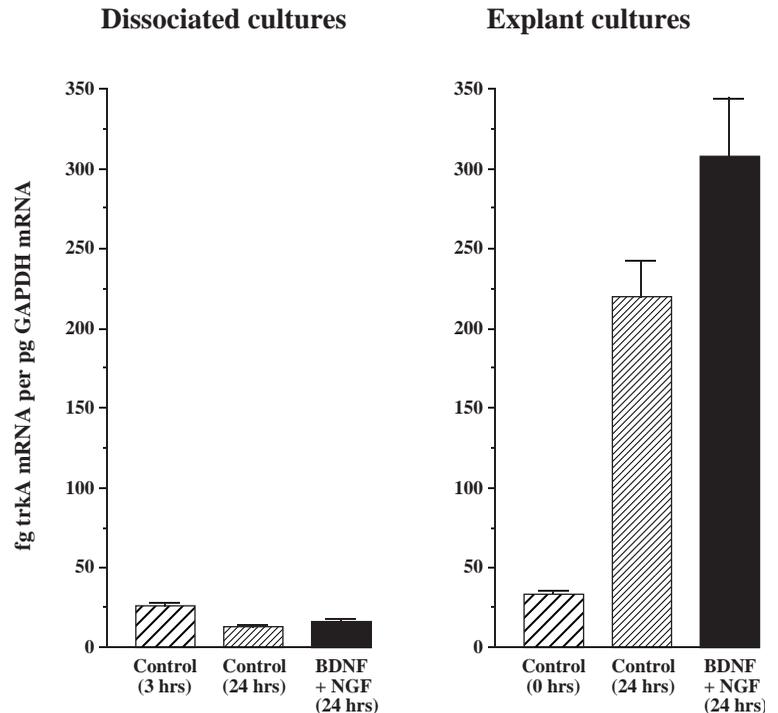
Dissociated cultures   **Explant cultures**

Fig. 5. Bar charts showing the levels of trkA mRNA relative to GAPDH mRNA in low-density dissociated cultures and explant cultures of E10.5 trigeminal ganglia. The initial measurements were carried out either 3 hours after plating (dissociated cultures) or immediately after dissection (explants) and were made after 24 hours incubation in neurotrophin-free medium (control) or in medium containing 5 ng/ml BDNF plus 5 ng/ml NGF (BDNF + NGF). The mean and s.e.m. of the results of two separate experiments each set up in duplicate are shown.

provide direct experimental evidence addressing this key question, we used repeated BrdU administration in utero to label all neurons generated in the trigeminal ganglion after an early stage in neurogenesis and studied the neurotrophin responsiveness of the unlabelled (early-born) neurons in cultures established after neurogenesis had ceased. In these latter cultures, the early-born neurons survived much better with NGF than BDNF, which is in marked contrast to the prominent BDNF survival response and poor NGF responsiveness of early-born neurons placed in culture shortly after they become postmitotic. This indicates that many early-born neurons that are initially supported by BDNF subsequently lose responsiveness to this neurotrophin and become NGF dependent. Thus, two changes occurring within the developing trigeminal ganglion account for the switch in neurotrophin responsiveness from BDNF to NGF observed in cultures established at sequential stages of development. First, neurons that respond initially to these different neurotrophins are generated during sequential, overlapping phases of development. Second, many of the neurons that initially respond to BDNF early in development subsequently lose responsiveness to BDNF and become dependent on NGF for survival.

We have also shown that many early-born trigeminal neurons switch responsiveness from BDNF to NGF when incubated for 24 hours in a trigeminal ganglion explant. Although cell proliferation continues in these explants and NGF-dependent neurons are born during this time, we were able to show that many of the neurons born prior to explantation had switched responsiveness from BDNF to NGF in the explant. In contrast, our previous studies have shown that when early trigeminal neurons are grown in dissociated cultures from the outset they retain dependence on BDNF well beyond the time when many of them would have become dependent on NGF for survival in vivo, suggesting that some signal that acts on the neurons in vivo to promote this switch

(Paul and Davies, 1995). The results of our explant cultures therefore suggest that this putative signal acts within the ganglion. The tissues innervated by the trigeminal neurons do not apparently play a role because the switch takes place in trigeminal ganglion explants whether or not the peripheral and central target field are included with the initial explant.

In summary, we demonstrate that there overlapping phases in the generation of BDNF- and NGF-responsive neurons in the developing trigeminal ganglion and provide the first conclusive evidence that some neurons switch their responsiveness in vivo from one neurotrophin to another during development. Furthermore, we show that the switch from BDNF to NGF responsiveness in these neurons is promoted by a locally acting signal within the ganglion. In future work it will be interesting to ascertain if this putative signal requires direct cell-cell interaction or if it is mediated by a diffusible agent, and whether it originates from neurons, precursor cells or other cells in the ganglion.

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