Sympathetic neuron survival and TrkA expression in NT3-deficient mouse embryos

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Several in vitro and in vivo studies have led to the widely accepted view that NT3 is required for sympathetic neuroblast survival, induction of TrkA expression and the acquisition of NGF dependence. However, we show that the number of neurons and the levels of trkA and p75 mRNAs in the superior cervical sympathetic ganglion (SCG) of NT3-/- mouse embryos increase normally up to E16, 2 days after SCG neurons start responding to NGF. At E18 and in the postnatal period, there are significant reductions in the number of SCG neurons and in the levels of trkA and p75 mRNAs. These results show that the neurotrophin survival requirements of SCG neurons do not switch from NT3 to NGF during development and that NT3 is not required for the expression of TrkA and p75 and the acquisition of NGF dependence. Rather, some sympathetic neurons have a late requirement for NT3 at the time when they also depend on NGF for survival. The expression of transcripts encoding catalytic TrkC is negligible at this stage, suggesting that NT3 acts mainly via TrkA.

Keywords: development/nerve growth factor/neurotrophins/superior cervical sympathetic ganglion/TrkA expression

Introduction

Nerve growth factor (NGF) is the founder member of a family of secreted proteins termed neurotrophins that promote and regulate the survival of neurons in the developing peripheral nervous system (Davies, 1994; Lewin and Barde, 1996). Experimental manipulation of NGF levels during development (Johnson et al., 1980; Levi-Montalcini, 1987) and studies of mice with null mutations in the NGF gene (Crowley et al., 1994) and NGF receptor tyrosine kinase (trkA) gene (Smye et al., 1994) have clearly shown that NGF is required for the survival of developing sympathetic neurons and a subset of sensory neurons. In the absence of NGF, these neurons are eliminated shortly after they begin innervating their target tissues from which they would normally obtain NGF. In addition to this requirement for target-derived NGF, several recent studies suggest that NGF-dependent sensory and sympathetic neurons require other neurotrophins for survival at an earlier stage in their development.

The sensory neurons of the trigeminal ganglion are supported by either brain-derived neurotrophic factor (BDNF) or neurotrophin-3 (NT3), but not NGF, in cultures set up at the stage when the earliest axons are growing to their peripheral targets, whereas later in development the neurons lose BDNF/NT3 responsiveness and become dependent on NGF for survival (Buchman and Davies, 1993; Paul and Davies, 1995). Accordingly, the number of neurons undergoing apoptosis in the trigeminal ganglion is markedly increased in embryos with null mutations in the BDNF receptor tyrosine kinase (trkB) and NT3 genes during the early stage of development when the neurons are supported by BDNF and NT3 in vitro, whereas neuronal apoptosis is markedly elevated in trkA-/- embryos later in development when neurons are supported by NGF in vitro (Piñón et al., 1996; Wilkinson et al., 1996).

During the early stages of their formation, paravertebral sympathetic ganglia contain many dividing cells that exhibit a variety of neuronal characteristics (Cohen, 1974; Rothman et al., 1978, 1980; Anderson and Axel, 1986; Rohrer and Thoenen, 1987; DiCicco-Bloom and Black, 1988; DiCicco-Bloom et al., 1990). Although many of these proliferating immature neurons or neuroblasts initially survive without added neurotrophins in culture (Ernsberger et al., 1989; Wyatt and Davies, 1995), a number of in vitro studies have shown that NT3, but not NGF, is capable of enhancing their survival (Birren et al., 1993; Dechant et al., 1993; DiCicco-Bloom et al., 1993). In cultures set up later in development when the neurons have begun to innervate their targets, most of the neurons are supported by NGF and only high levels of NT3 are effective in promoting survival (Birren et al., 1993; Dechant et al., 1993). In accordance with the early in vitro survival response of sympathetic neuroblasts to NT3, early sympathetic ganglia express high levels of trkC mRNA which encodes the NT3 receptor tyrosine kinase (Birren et al., 1993; DiCicco-Bloom et al., 1993; Fagan et al., 1996) and are surrounded by non-neuronal cells expressing NT3 mRNA (Verdi et al., 1996). At later embryonic stages, trkC mRNA expression declines (Birren et al., 1993; DiCicco-Bloom et al., 1993; Fagan et al., 1996), the expression of trkA mRNA increases (Birren et al., 1993; Verdi and Anderson, 1994; Wyatt and Davies, 1995) and NGF is synthesized in the tissues innervated by sympathetic neurons (Korschning and Thoenen, 1988).

The above studies have led to the widely accepted view that sympathetic neuroblasts are transiently dependent on locally produced NT3 for survival before becoming dependent on target-derived NGF. Furthermore, because NT3 increases trkA mRNA expression in sympathetic neuroblast cultures, it has been concluded that NT3 induces TrkA expression and NGF responsiveness in developing...
sympathetic neurons (Verdi and Anderson, 1994; Verdi et al., 1996). The generation of mice with targeted null mutations in the NT3 gene (Ernfors et al., 1994; Farinas et al., 1994) has made feasible a rigorous examination of the physiological relevance of these assumptions. We show that the number of neurons and the levels of transcripts encoding NGF receptors increase normally in NT3-deficient embryos for several days after the earliest neurons start responding to NGF. Significant neuronal deficiencies in NT3-deficient mice are only observed late in development, indicating a late, not early, requirement for NT3. Furthermore, although trkC mRNA levels are high in early SCG, the most prominent transcripts by far are those encoding non-catalytic receptors that lack a kinase domain.

Results

Neuronal deficiencies appear late in the SCG of NT3–/– mice

The total number of neurons in the SCG at three stages of development was estimated by counting neuronal profiles in 8 µm serial sections (Figure 1). At E16, which is 2 days after the earliest SCG neurons start responding to NGF in vitro (Wyatt and Davies, 1995), there was no significant difference in the number of neurons in the SCG of wild-type and NT3–/– embryos (P = 0.28, n = 15, t-test). At E18, there were significantly fewer (19%) neurons in the SCG of NT3–/– embryos (P = 0.015, n = 16, t-test), and this difference widened to 28% by P1 (P = 0.0008, n = 15, t-test).

To provide an indication of the number of dying cells at each of these ages, the total number of pyknotic nuclei was also counted in the serially sectioned SCG (Figure 1). Pyknotic nuclei were recognized as one or more very darkly stained spherical structures contained within a clearly visible membrane (Piñón et al., 1996). The great majority of these pyknotic nuclei were observed in large degenerating cells, suggesting that these were neurons (Oppenheim, 1991). There were no significant differences in the number of pyknotic nuclei between wild-type and NT3–/– embryos at E16 (P = 0.09, n = 16, t-test). At E18 and P1 there were significantly more (46% and 60%, respectively) pyknotic nuclei in the SCG of NT3–/– embryos (P < 0.01, n = 16, t-tests).

To provide an additional measure of the stage at which the NT3 null mutation affects SCG development, we used competitive RT–PCR to quantify the levels of mRNAs encoding two ubiquitous, constitutively expressed ‘housekeeping’ proteins, the L27 ribosomal protein and glyceraldehyde phosphate dehydrogenase (GAPDH). These measurements were carried out on total RNA extracted from both SCG dissected from three to seven embryos and neonates of each genotype at each stage. In the SCG of wild-type mice, the levels of these mRNAs increased in parallel from E14 to P1 (Figure 2), reflecting the increase that occurs in the total number of cells in the SCG (neurons plus other cell types) over this period of development. At E14, E15 and E16, there were no significant differences in the levels of these mRNAs between wild-type and NT3–/– embryos (P > 0.2 in all cases, t-tests). By E18, there were significantly lower levels of L27 mRNA (18% lower, P = 0.004, n = 12, t-test) and GAPDH mRNA (14% lower, P = 0.035, n = 12, t-test) in the SCG of NT3–/– embryos. By P1, the levels of L27 and GAPDH mRNAs were 39% and 37% lower, respectively (P < 0.02, n = 11, in each case). These findings support the results of our histological analysis which suggest that there are no significant differences in the number of neurons in the SCG of NT3–/– and wild-type embryos at E16, but that at E18 and later stages there are fewer neurons in the SCG of NT3-deficient mice. In addition, these findings also suggest that numbers of other cell types in the SCG are unaffected by NT3 during the early stages of gangliogenesis.

![Fig. 1. Total number of pyknotic nuclei and total number of neurons in the SCG of wild-type (NT3+/+) and NT3–/– mice at E16, E18 and P1. The means and standard errors of the mean of either seven or eight animals of each genotype at each age are shown.](image-url)
and the level of p75 mRNA was 55% lower ($P = 0.0003$, $n = 12$, t-test). At P1, the level of trkA mRNA was 35% lower in the SCG of NT3−/− mice ($P = 0.03$, $n = 11$, t-test) and the level of p75 mRNA was 53% lower ($P = 0.003$, $n = 10$, t-test).

There was no significant difference between the levels of trkA mRNA relative to L27 mRNA in the SCG throughout development ($P > 0.1$ at each stage, t-tests), suggesting that the reduction in the absolute level in trkA mRNA in the SCG of NT3−/− mice at E18 and P1 is due to the reduced number of neurons. However, the level of p75 mRNA relative to L27 mRNA in the SCG of E18 and P1 mice was significantly lower than that in wild-type mice ($P > 0.02$, $n = 12$ and 10, respectively, t-tests). This suggests that, in addition to playing a role in maintaining the survival of a proportion of the neurons in the SCG in late fetal and neonatal stages, endogenous NT3 plays a role in maintaining the level of p75 mRNA in SCG cells at these late stages.

To determine if the lack of effect of NT3 on the regulation of trkA mRNA and p75 mRNA expression is a peculiar feature of the SCG, we carried out a limited number of studies on the expression of these mRNAs in other ganglia of the paravertebral sympathetic chain. The stellate ganglion and ganglia of the brachial part of the sympathetic chain were dissected from E16 wild-type and NT3−/− embryos. There was no significant difference in the levels of trkA mRNA in wild-type (0.327 ± 0.026 pg trkA mRNA per pg GAPDH mRNA, mean ± SEM) and NT3−/− (0.285 ± 0.029 pg trkA mRNA per pg GAPDH mRNA) embryos ($P = 0.19$, $n = 9$, t-test). Likewise, there was no significant difference in the levels of p75 mRNA in wild-type (0.207 ± 0.029 pg p75 mRNA per pg GAPDH mRNA, mean ± SEM) and NT3−/− (0.172 ± 0.024 pg p75 mRNA per pg GAPDH mRNA) embryos ($P = 0.2$, $n = 9$, t-test).

To further investigate if endogenously synthesized NT3 has any role in regulating trkA mRNA expression, and to examine the possibility that NT3 could be acting by an autocrine route in vitro, we established cultures of SCG neuroblasts from wild-type and NT3−/− embryos at E14, the stage at which we had previously shown that the level of trkA mRNA increases in neuroblasts cultured in defined medium without added NT3 (Wyatt and Davies, 1995). In E14 cultures established from embryos of both genotypes, the majority of neurons survived for 48 h in defined medium containing NGF. As shown in Figure 4, the level of trkA mRNA increased to a similar extent between 24 and 48 h in cultures established from both wild-type embryos (from 0.500 ± 0.057 to 1.439 ± 0.079, $n = 4$ at each time point, significant increase, $P = 0.0001$, t-test) and NT3−/− embryos (from 0.408 ± 0.052 to 1.476 ± 0.157, $n = 6$ at each time point, significant increase, $P = 0.0001$, t-test). This indicates that NT3 is not required for the induction of trkA mRNA expression in culture.

Expression of trkC transcripts in developing SCG

Alternative splicing generates transcripts that encode TrkC variants that lack the catalytic tyrosine kinase domain (Lamballe et al., 1993; Tsoulfas et al., 1993; Valenzuela et al., 1993). The levels of mRNAs encoding catalytic (TK+) and non-catalytic (TK−) TrkC variants in developing SCG were measured by competitive RT–PCR using

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**Fig. 2.** Levels of L27 mRNA and GAPDH mRNA in the SCG of NT3+/+ and NT3−/− mice at stages from E14 to P1. The means and standard errors of the mean of between three and seven animals of each genotype at each age are shown.

**L27 mRNA**

- NT3 +/+ (solid line)
- NT3 −/− (dotted line)

**GAPDH mRNA**

- NT3 +/+ (solid line)
- NT3 −/− (dotted line)

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**trkA and p75 mRNAs levels initially increase normally in NT3-deficient embryos**

Competitive RT–PCR was used to measure the levels of trkA mRNA and p75 mRNA in SCG dissected from wild-type and NT3−/− embryos at stages from E14 to P1. As reported previously (Wyatt and Davies, 1995), the absolute levels of these mRNAs increased in the SCG of wild-type mice during this period of development (Figure 3). The levels of trkA and p75 mRNAs increased normally in the SCG of NT3−/− embryos up to E16, at which stage there was no significant difference between the levels of these mRNAs in wild-type and NT3−/− embryos ($P > 0.1$, $n = 9$, in each case, t-tests). At later ages, however, the levels of these mRNAs were significantly lower in NT3−/− mice. At E18, the level of trkA mRNA was 30% lower in the SCG of NT3−/− mice ($P = 0.005$, $n = 12$, t-test) and the level of p75 mRNA was 55% lower ($P = 0.0003$, $n = 12$, t-test). At P1, the level of trkA mRNA was 35% lower in the SCG of NT3−/− mice ($P = 0.03$, $n = 11$, t-test) and the level of p75 mRNA was 53% lower ($P = 0.003$, $n = 10$, t-test).

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Fig. 3. Levels of trkA mRNA and p75 mRNA (absolute levels per ganglion and levels relative to the level of L27 mRNA) in the SCG of NT3⁺/− and NT3−/− mice at stages from E14 to P1. The means and standard errors of the mean of between three and seven animals of each genotype at each age are shown.

The levels of TK+/H11001 trkC mRNA in the SCG were low during the early stages of its development and fell with increasing age, becoming negligible by E18 (Figure 5). In contrast, the levels of TK− trkC mRNA were substantially higher. The expression of TK− trkC mRNA was highest (relative to GAPDH mRNA) between E13 and E15. During this period of development, the level of TK− trkC mRNA was 30- to 40-fold higher than that of TK+ trkC mRNA. The level of TK− trkC mRNA decreased with age, whereas the level trkA mRNA increased. By P1, the level of trkA mRNA was ~2-fold higher than that of TK− trkC mRNA (Figure 5).

To determine if the high levels of TK− trkC mRNA primers specific for mouse TK+ transcripts and primers in the extracellular domain that amplify all mouse trkC variants. It is not possible to design a single RT–PCR assay to measure all of the mRNAs that encode TK− trkC variants directly because there is no exonic sequence common to and specific for all TK− trkC mRNAs (Valenzuela et al., 1993). The level of TK− mRNAs was therefore calculated by subtracting TK+/H11001 mRNA from total trkC mRNA.

Fig. 4. Autoradiograph showing the products of RT–PCR reactions using trkA-specific primers to determine the levels of trkA mRNA in E14 SCG neurons from wild-type and NT3−/− embryos after 24 and 48 h in culture. The products of trkA mRNA (75 bp) and the trkA competitor cRNA (78 bp) are indicated (20 fg of trkA competitor cRNA were added to all reactions). Lanes 1–4 show the products of reactions using total RNA from 300 sympathetic neurons of two wild-type embryos (A and B) cultured for 24 h (lanes 1 and 2) and 48 h (lanes 3 and 4). Lanes 7–10 show the products of reactions using total RNA from 300 sympathetic neurons of two NT3−/− embryos (C and D) cultured for 24 h (lanes 7 and 8) and 48 h (lanes 9 and 10). Lanes 5, 6, 11 and 12 are control reactions (cont) showing that no product was obtained in the absence of reverse transcriptase.
in developing SCG are expressed in part by neurons, differential sedimentation (Davies, 1986) was used to separate neurons from other cell types. This separation technique was carried out at P1 when the size difference between neurons and non-neuronal cells in the ganglion was large enough to obtain neuronal preparations of > 95% purity. Substantial levels of TK^{+} trkC mRNA were measured in these neurons (586 ± 9.6 fg TK^{+} trkC mRNA per pg GAPDH mRNA), whereas the level of TK^{-} trkC mRNA was 3000-fold lower (0.195 ± 0.011 fg TK^{-} trkC mRNA per pg GAPDH mRNA).

To investigate if endogenous NT3 plays a role in regulating the levels of TK^{+} and TK^{-} trkC mRNAs in developing SCG in vivo, the levels of these mRNAs were measured in the SCG of wild-type and NT3^{-/-} mice throughout development. The levels of these mRNAs relative to GAPDH mRNA were very similar in both genotypes (Figure 6), suggesting that NT3 does not play a role in regulating trkC mRNA expression in developing sympathetic neurons.

To determine if the levels of TK^{+} trkC mRNA were also high relative to TK^{+} trkC mRNA in other ganglia of the sympathetic chain, the levels of these mRNAs were measured in stellate ganglion and ganglia of the brachial part of the sympathetic chain of E16 embryos. As in the SCG, the level of TK^{+} trkC mRNA was substantially higher (1730 ± 216 fg TK^{+} trkC mRNA per pg GAPDH mRNA) than that of TK^{-} trkC mRNA (2.96 ± 0.45 fg TK^{-} trkC mRNA per pg GAPDH mRNA).

Discussion

We have demonstrated by several criteria that the cellular composition of the SCG is unaffected by the absence of endogenous NT3 during the early stages of development when sympathetic neurons have started to respond to NGF. By E16, when the majority of neurons have become responsive to NGF in vitro (Wyatt and Davies, 1995), there are no significant differences in the total number of neurons and in the number of cells undergoing apoptosis in the SCG of wild-type and NT3-deficient embryos. There are also no significant differences in the levels of mRNAs encoding two ‘housekeeping’ proteins, L27 and GAPDH, in the SCG of wild-type and NT3-deficient embryos at E16, indicating that there are similar total numbers of cells in the SCG at this stage. Thus, contrary to the view based on in vitro studies that sympathetic neuroblasts are dependent on NT3 for survival before becoming NGF-dependent (Birren et al., 1993; DiCicco-Bloom et al., 1993), our results demonstrate that NT3 is not required for the survival of SCG neuroblasts in vivo before they acquire NGF responsiveness. Although it has been reported that there are significantly fewer neurons and significantly higher numbers of apoptotic cells in the SCG of NT3^{-/-} embryos at stages prior to E16 (ElShamy et al., 1996), a relatively small number of embryos was analysed, which may have produced spurious results because of variability between embryos. In contrast, over 60 embryos of each genotype were analysed in our current study. Furthermore, a recent analysis of newly generated NT3^{-/-} embryos in the Karolinska Institute has failed to detect any significant neuronal losses in the SCG at E14 and E17 (P. Ernfors, unpublished results). Thus, we conclude that SCG neurons are not lost in the early development of NT3^{-/-} mice, and that the early loss previously reported (ElShamy et al., 1996) is either erroneous or due to a different genetic background.

Our study also demonstrates that endogenous NT3 is not required for induction of TrkA and p75 mRNA expression in sympathetic neuroblasts. The levels of these mRNAs increase normally in the sympathetic ganglia of NT3-deficient embryos during the period when the neurons are acquiring NGF dependence. By E16, when most of the neurons have become dependent on NGF for survival, the levels of these mRNAs in the SCG and other ganglia of the sympathetic chain are not significantly different in wild-type and NT3-deficient embryos. Furthermore, trkA mRNA expression in E14 sympathetic neuroblasts increases to the same extent in cultures established from
wild-type and \( NT3^{-/-} \) embryos in defined medium without NT3. The view that NT3 plays a key role in inducing TrkA expression in sympathetic neuroblasts was based on the finding that NT3 increases \( trkA \) mRNA expression in sympathetic neuroblasts in vitro (Verdi and Anderson, 1994; Verdi et al., 1996). It is notable, however, that very high concentrations of NT3 were needed to induce \( trkA \) mRNA expression in these experiments. The required levels of NT3 were, for example, four orders of magnitude higher than the level of NT3 that promotes the in vitro maturation of early sensory neurons (Wright et al., 1992).

Although we have excluded a role for endogenous NT3 in the generation and survival of sympathetic neuroblasts in the SCG and have shown that induction of \( trkA \) expression and NGF responsiveness occur independently of NT3, our results demonstrate a late requirement for NT3 in sympathetic neuron development. At E18, there are significantly fewer neurons and correspondingly lower levels of L27 and GAPDH mRNAs in the SCG of NT3-deficient embryos. This neuronal deficiency results from increased cell death because the number of apoptotic cells in the SCG is significantly higher in NT3-deficient embryos at this stage. A further reduction in neuron number and increase in apoptosis were evident in P1 NT3-deficient neonates compared with wild-type neonates, suggesting that an increasing proportion of sympathetic neurons require NT3 for survival during the late embryonic and early postnatal period. Previous estimates of the number of neurons in the SCG of NT3\(^{-/-}\) mice carried out in the second postnatal week have shown that there are about half the normal number (Ernfors et al., 1994; Farinas et al., 1994), suggesting that further losses of sympathetic neurons occur throughout the postnatal period in the absence of NT3. Because of the results of previous in vitro studies (Birren et al., 1993; Dechent et al., 1993; DiCicco-Bloom et al., 1993), it was assumed that the neuronal deficiencies in the SCG of NT3\(^{-/-}\) postnatal mice had resulted from the loss of sympathetic neuroblasts (Ernfors et al., 1994; Farinas et al., 1994). However, our current results indicate that the neuronal deficiencies in the SCG of NT3\(^{-/-}\) postnatal mice result from an increased loss of neurons that does not begin until the late embryonic period. Furthermore, the demonstration that administration of a specific anti-NT3 antiserum to neonatal rats results in the death of 60–80\% of SCG neurons (Zhou and Rush, 1995) suggests that many SCG neurons are dependent on endogenous NT3 for survival during the postnatal period. Administration of anti-NGF antiserum during this period virtually eliminates sympathetic neurons (Levi-Montalcini and Booker, 1960) and the SCG is virtually absent by P10 in \( NGF^{-/-} \) and \( trkA^{-/-} \) mice (Crowley et al., 1994; Smeyne et al., 1994). Thus, both NT3 and NGF seem to play a role in regulating the survival of sympathetic neurons during the period of naturally occurring cell death which largely takes place postnatally (Wright et al., 1983).

Although our results suggest that NT3 is neither required for the induction of \( trkA \) and p75 expression in sympathetic neuroblasts nor for the maintenance of \( trkA \) levels in SCG neurons at later stages of development, our finding that the level of p75 mRNA relative to L27 mRNA in the SCG of late fetal and neonatal NT3-deficient mice is significantly lower than that in wild-type mice raises the possibility that endogenous NT3 may play a role in maintaining the appropriate level of p75 expression in older SCG neurons. It is possible, however, that the relative reduction in p75 levels in the SCG of NT3-deficient mice at these late developmental stages may reflect changes in p75 expression in non-neuronal cells within the ganglion.

Previous studies have shown that high levels of \( trkC \) mRNA are expressed in early sympathetic ganglia and that with increasing age the level of \( trkC \) mRNA decreases (Birren et al., 1993; DiCicco-Bloom et al., 1993; Fagan et al., 1996; Verdi et al., 1996). Although these observations have been interpreted as supporting evidence for the survival dependence of sympathetic neuroblasts on NT3, the TrkC ligand (Birren et al., 1993; DiCicco-Bloom et al., 1993; Verdi et al., 1996), the kinds of \( trkC \) transcripts expressed in developing sympathetic ganglia were not ascertained. We show that \( trkC \) transcripts encoding truncated, non-catalytic receptors outnumber those encoding tyrosine kinase-containing receptors by 30- to 40-fold in early sympathetic ganglia and that by late embryonic stages, when NT3 starts to play a role in promoting sympathetic neuron survival, the level of \( trkC \) mRNA encoding catalytic receptors is negligible. From assays carried out on RNA extracted from purified postnatal sympathetic neurons we show that these neurons express \( trkC \) mRNA encoding truncated receptors, and because the vast majority of cells in early sympathetic ganglia are proliferating sympathetic neuroblasts (Rohrer and Thoenen, 1987), it is likely that these cells also express appreciable levels of \( trkC \) mRNA encoding truncated receptors. Because Trk receptors possessing a kinase domain mediate the survival-promoting effects of neurotrophins (Barbacid, 1994; Kaplan and Stephens, 1994; Klein, 1994) and truncated receptors function as negative modulators of neurotrophin signalling in neurons (Ninkina et al., 1996) and other cell types (Eide et al., 1996), these results suggest that the late requirement of sympathetic neurons for NT3 is not mediated by its preferred receptor TrkC. The demonstration that there are no statistically significant differences between the number of neurons in the SCG of \( trkC^{-/-} \) and wild-type mice throughout development (Fagan et al., 1996) likewise indicates that TrkC receptors are not required for sympathetic neuron survival. Because NT3 can promote the in vitro survival of the majority of late embryonic and postnatal SCG sympathetic neurons obtained from \( trkC^{-/-} \) mice (Davies et al., 1995a), it is likely that NT3 exerts its effects on late embryonic and postnatal sympathetic neurons \textit{in vivo} by signalling via TrkA. Although TrkC receptors are not apparently involved in promoting the survival of developing sympathetic neurons, it is possible that they may play a role in regulating other aspects of sympathetic neuron development. Studies of neurogenesis in the DRG of NT3\(^{-/-}\) embryos suggest that NT3 plays a role in keeping precursor cells in the proliferative state and prevents their differentiation (Farinas et al., 1996). However, because the cellular composition of the SCG is unaffected in NT3-deficient embryos during the period of gangliogenesis it seems unlikely that endogenous NT3 regulates cell cycle parameters in sympathetic neuroblasts.

Although it has been shown previously that NT3 increases the expression of \( trkC \) mRNA in postnatal sympathetic neurons in culture (ElShamy et al., 1996),
we show that the expression of trkC transcripts encoding both catalytic and non-catalytic receptors is regulated independently of endogenous NT3. Likewise, although several studies have shown that NGF administration increases trkA expression both in vivo and in vitro (Holtzman et al., 1992; Kojima et al., 1994; Sohrabi et al., 1994; Venero et al., 1994), studies of NGF−/−embryos have shown that trkA mRNA expression in at least developing sensory neurons is regulated independently of NGF (Davies et al., 1995b). Thus, neurotrophins appear to play a less prominent role in regulating the in vivo expression of their receptors during development than would have been predicted from previous studies.

In summary, we have demonstrated that in the developing SCG, NT3 is not required for the survival of sympathetic neuroblasts and is not needed for the induction of trkA mRNA expression and the onset of NGF dependence. However, later in development, an increasing proportion of neurons becomes dependent on NT3 for survival and NT3 at this stage, like NGF, exerts its effects its survival-promoting effects via TrkA. Thus, sympathetic neuroblasts in the SCG do not switch dependence from NT3 to NGF during development, but acquire an additional requirement for NT3 after they have become NGF-dependent, and both factors seem to play a role in regulating survival during the period of naturally occurring neuronal death. Although we have focused primarily on the developing SCG in our current study, there appear to be some differences in the developmental requirements of the rostral and caudal parts of the sympathetic chain. For example, whereas the SCG is eliminated in ret−/− mice by birth (Durbec et al., 1996) and has significantly fewer neurons in GDNF−/− mice (Moore et al., 1996), the more caudal parts of the sympathetic chain are apparently unaffected by either of these null mutations. Thus, it may be informative to carry out detailed developmental studies of other ganglia of the sympathetic chain in NT3 mutant mice.

Materials and methods

Experimental animals

Embryos were obtained from overnight matings of NT3+/+ mice (Emfors et al., 1994). Pregnant females were killed by cervical dislocation at the required stage of gestation and the precise stage of development of the embryos was determined by the criteria of Theiler (1972). DNA was extracted from embryonic tissues to determine the genotypes of embryos by PCR using primers specific for the wild-type and mutated NT3 genes.

Neuron cultures

Separate dissociated cultures of SCG neurons were set up from each embryo (Davies et al., 1995a). The neurons were grown in 35 mm diameter plastic tissue culture wells (Nunc) that had been previously coated with polyornithine (0.5 mg/ml, overnight) and laminin (20 μg/ml for 4 h). The neurons were plated at a density of ~500–2000 neurons per ml of Ham’s F14 supplemented with 2 mM glutamine, 0.35% bovine serum albumin (Pathocyte-4, ICN), 60 ng/ml progesterone, 16 μg/ml putrescine, 400 ng/ml t-thyroxine, 38 ng/ml sodium selenite, 340 ng/ml tri-iodo-thyronine, 60 ng/ml penicillin and 100 mg/ml streptomycin. All cultures also received NGF at a concentration of 5 ng/ml (gift of John Winslow and Gene Burton of Genentech, Inc.). The cultures were incubated at 37°C in a 5% CO2 incubator.

Quantification of total numbers of neurons and pyknotic nuclei in the SCG

The SCG were carefully dissected from E16 and E18 embryos and P1 neonates and were inserted in a piece of liver before fixation in 4% paraformaldehyde in 0.1M phosphate buffer, pH 7.3. After fixation for at least a week, the tissue was dehydrated and embedded in paraffin wax and serially sectioned at 8 μm. The sections were mounted on either gelatinized slides or polylysine-coated slides and were stained with cresyl fast violet.

To estimate the total number of neurons in the SCG, 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 1000× 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).

To evaluate the extent of cell death, all pyknotic nuclei were counted at 400× magnification in every fourth section along the entire rostro-caudal extent of the SCG at E16, E18 and P1. Estimates of the total number of pyknotic nuclei in each ganglion were obtained by multiplying the sum of these counts by four. In all cases, the sections were coded before counting, to avoid any observer bias.

Quantification of neurotrophin receptor mRNA levels

A quantitative RT–PCR technique (Wyatt and Davies, 1993) was used to measure the levels of trkA, trkC, p75, GAPDH and L27 mRNAs in total RNA extracted from neuronal cultures and sympathetic ganglia of wild-type and NT3−/− mice. The reverse transcription and PCR reactions were calibrated by the inclusion of known amounts of cRNA competitor templates for each of the mRNAs in the reverse transcription reaction. The cRNA competitor templates were synthesized in vitro from cDNA competitor constructs. Previous detailed comparisons of this method with quantitative Northern blotting has demonstrated accuracy and reproducibility over a wide range of concentrations of trkA and p75 mRNA (Wyatt and Davies, 1993).

In the rat, the cytoplasmic domain of the kinase-deficient (TK−) TrkC receptor is encoded by four different exons (Valenzuela et al., 1993). There are several TrkC TK− variants that contain different combinations of these exons. Because no one exon is consistently present in all of these variants, it is not possible to design a single RT–PCR assay to measure all mRNAs that code for trkC TK− receptors. For this reason, we have calculated the levels of trkC TK− mRNAs by subtracting the level of trkC TK+ mRNA from the total amount of trkC mRNA encoding both the TK− and TK+ isoforms. The total amount of trkC mRNA was assayed by using an RT–PCR assay that is specific for the extracellular domain of the trkC mRNA. Although mRNAs for several minor TK+ and TK− TrkC variants that do not encode the extracellular domain have been identified in chicken (Garner and Large, 1994), these have not been described in mouse or rat. The expression of such mRNA isoforms is unlikely therefore significantly to affect our results.

The construction of trkA, p75 and L27 competitor cDNAs has been described previously (Wyatt and Davies, 1993, 1995). The GAPDH competitor cDNA was made by amplifying a 401 bp cDNA fragment by RT–PCR of RNA extracted from E14 mouse trigeminal ganglia. The forward primer was 5′-CCCTCTACGACCCTCAACTACATG-3′ and the reverse primer was 5′-GGCAATGACTTGGTGCATTCACT-3′. The cDNA fragment generated was cloned into pGEM t-vector (Promega). The cloned cDNA was cut at a unique HindIII site restriction site, the cohesive ends were filled using Klenow and the blunt ends were religated, resulting in a competitor cDNA that was 4 bp longer than the native cDNA.

The competitor cDNA used for calibrating the RT–PCR assay specific for the kinase domain of trkC was made using RT–PCR to amplify from E14 mouse trigeminal ganglion RNA a 539 bp trkC cDNA fragment that encompasses the juxtamembrane domain and much of the kinase domain. The forward primer was 5′-TGTCGCTGTATCATGGTGAGC-3′ and the reverse primer was 5′-GCTGACCGGACTGTCATC-3′. The cDNA fragment was cloned into pGEM t-vector (Promega). The cloned cDNA was cut at a unique HindIII site restriction site, the cohesive ends were filled using Klenow, and the resulting blunt ends were religated, resulting in a competitor cDNA that was 4 bp longer than the corresponding trkC mRNA.

The trkC competitor used to calibrate the RT–PCR assay specific for mRNAs encoding the extracellular domain of mouse trkC was made by isolating a 461 bp trkC cDNA fragment by RT–PCR. The forward primer was 5′-GCAAGAACAATCATGCTAGTG-3′ and the reverse primer was 5′-CTTCACTGACCCCTCAACTACATG-3′.
was 5'-CTGGTAGTAGTCATATGG-3'. The cloned cDNA (in pGEM t-vector) was cut at a unique BclI site, the cohesive ends were filled using Klenow and the blunt ends were religated, resulting in a competitor cDNA that was 4 bp longer than the corresponding trkC cDNA. Total RNA (Chomczynski and Sacchi, 1987), spiked with known amounts of the appropriate competitor cRNA produced by in vitro transcription from the competitor cDNA, was reverse transcribed for 45 min at 37°C using SuperScript reverse transcriptase (Gibco) in a 40 µl reaction containing the manufacturer's buffer supplemented with 0.5 mM dNTPs and 10 µM random hexanucleotides. A 0.5 µl aliquot of each reverse transcription reaction was then amplified in a 50 µl PCR reaction containing: 10 Promega PCR buffer, 1.5 mM MgCl₂, 0.1 mM dNTPs, 1.5 units of Taq polymerase (Promega), 40 ng of 32P-5' end-labelled primers specific for either trkA, trkC, p75, L27 or GAPDH. Assay primers and cycling conditions for amplifying trkA, p75 and L27 cDNAs have been described previously (Wyatt and Davies, 1993, 1995). The forward assay primer for amplifying GAPDH cDNA was 5'-TCCTAGTATGACTCCTAC-3' and the reverse primer was 5'-TCTTGGAGATGGTGTAGG-3'. These hybridize 128 bp apart in mouse GAPDH cDNA and 132 bp apart in the GAPDH competitor cDNA. In the case of trkC cDNAs, the choice of assay primers was constrained by the need to design primers that would hybridize only with trkC sequences and not to related trkA and trkB sequences. The choice of suitable primers was facilitated by sequence alignment and the specificity of primers was tested directly using trkA and trkB cDNAs as templates. The forward primer for assaying cDNAs encoding the kinase domain of trkC was 5'-CCCAACAAAGACAAGATG-3' and the reverse primer was 5'-CCAGAGATCAGTGTGAGC-3'. These primers hybridize 105 bp apart in mouse trkC cDNA and 72 bp apart in the trkC kinase domain competitor cDNA. These primers amplify all trkC cDNAs including those containing inserts in the kinase domain (Lamballe et al., 1995). The forward PCR primer for amplifying trkC cDNAs encoding the extracellular domain of trkC was 5'-CCAGGATCATGTTGAGCC-3' and the reverse primer was 5'-TATCCGATCCACATCGAGG-3'. These hybridize 105 bp apart in mouse trkC cDNA and 109 bp apart in the trkC extracellular domain competitor cDNA.

GAPDH cDNA and the cDNA encoding a region of the kinase domain of trkC were amplified by cycling at 95°C for 1 min, followed by 40 s at 53°C, followed by 75 s at 72°C. In the case of the cDNA encoding a region of the extracellular domain of trkC, the annealing step of the cycle was 52°C for 40 s. The exact number of PCR cycles was dependent on the initial target cDNA concentration but was typically between 25 and 30 cycles. The RT–PCR products of the native mRNAs and their corresponding cRNA competitor species were separated on 8% non-denaturing polyacrylamide gels that were subsequently dried and autoradiographed. Reactions were set up so that the intensity of the autoradiographic signals for the native mRNA and competitor cRNA RT–PCR products were approximately the same to avoid inaccuracies encountered due to the narrow linear response range of X-ray film. The intensities of the autoradiograph signals from the RT–PCR products of the native mRNA and competitor RNA were calculated using a laser densitometer (Molecular Dynamics). The amount of trkA, trkC, p75, L27 and GAPDH mRNAs present in the initial total RNA samples were calculated from these values.

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References


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