p75-deficient embryonic dorsal root sensory and neonatal sympathetic neurons display a decreased sensitivity to NGF

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SUMMARY

To understand the role of low-affinity neurotrophin receptor p75 in neural development, we previously generated mice carrying a null mutation in the p75 locus (Lee, K. F., Li, E., Huber, L. J., Landis, S. C., Sharpe, A. H., Chao, M. V. and Jaenisch, R. (1992) Cell 69, 737-749). To elucidate the mechanisms leading to deficits in the peripheral nervous system in p75 mutant mice, we have employed dissociated cultures to examine the responses of p75-deficient dorsal root ganglion (DRG) and superior cervical ganglion (SCG) neurons to different neurotrophins. We found that p75-deficient DRG and SCG neurons displayed a 2- to 3-fold decreased sensitivity to NGF at embryonic day 15 (E15) and postnatal day 3 (P3), respectively, ages that coincide with the peak of naturally occurring cell death. Furthermore, while p75-deficient E15 DRG neurons did not change their response specificity to BDNF, NT-3, and NT-4/5, P3 SCG neurons became more responsive to NT-3 at higher concentrations (nanomolar ranges). These results may help explain the deficits in the peripheral nervous system in p75 mutant mice and provide evidence that p75 can modulate neurotrophin sensitivity in some neurons.

Key words: p75, neurotrophins, survival, mouse

INTRODUCTION

Nerve growth factor (NGF; Levi-Montalcini, 1987) is a member of a gene family of neurotrophic factors (neurotrophins) that includes brain-derived neurotrophic factor (BDNF; Barde et al., 1982; Leibrock et al., 1989), neurotrophin-3 (NT-3; Hohn et al., 1990; Maisonpierre et al., 1990; Rosenthal et al., 1990; Ernfors et al., 1990; Jones and Reichardt, 1990) and neurotrophin-4/5 (NT-4/5; Berkemeier et al., 1991; Hallböök et al., 1991; Sutter et al., 1979). Two classes of cell surface receptors have been described: all neurotrophins bind to p75 with low affinity (Johnson et al., 1986; Radeke et al., 1987; Ernfors et al., 1990; Rodriguez-Tébar et al., 1992) and to specific tyrosine receptor kinases (trkA, trkB, and trkC) with higher affinity (Barbacid et al., 1991; Cordon-Cardo et al., 1991; Kaplan et al., 1991; Klein et al., 1991a,b; Lamballe et al., 1991; Soppet et al., 1991; Squinto et al., 1991; Chao, 1992). Experiments utilizing fibroblasts expressing the individual trks indicate that trkA is the cognate receptor for NGF, trkB for BDNF and NT-4/5, and trkC for NT-3, although there is a certain degree of ligand-receptor promiscuity (Chao, 1992; Meakin and Shooter, 1992). In contrast to the wide expression of p75 (Bothwell, 1991), trks have a more restricted pattern of expression (Klein et al., 1990; Martin-Zanca et al., 1990; Ernfors et al., 1992) and are essential for neurotrophin signal transduction. Recently, Klein et al. (1993) showed that mice homozygous for a mutation in the trkB receptor gene exhibit deficits in central and peripheral nervous system.

The contribution, if any, of p75 to neurotrophin signal transduction in neurons is not clear. There is a major controversy over whether high-affinity NGF binding and signal transduction are dependent solely on the expression of trkA or whether co-expression of trkA and p75 is required. While Klein et al.
et al., 1992). Mutant animals also display tissue-specific and the development of skin ulcerations in the extremities (Lee periphery, which is associated with decreased heat sensitivity decreased cutaneous sensory innervation by calcitonin-gene p75 locus by gene-targeting in embryonic stem cells (Lee et al., 1992). Mutant mice are viable and exhibit markedly previously generated mutant mice carrying a null mutation in the through its influence on the migration of developing Schwann results suggest a role for p75 in neuronal patterning nerves (Johnson et al., 1988) and in target fields prior to and p75 is expressed on Schwann cells in regenerating peripheral variety of functions in non-neuronal cells. The observation that Embryos were obtained from timed-pregnant mice and staged by the Animals normally shifts to higher NGF concentrations with age this domain remains to be tested. Furthermore, although all neurotrophins bind p75 with similar affinity, their dissociation rates are markedly different; NGF and NT-3 being the fastest and slowest, respectively (Rodriguez-Têbar et al., 1992). This difference raises the possibility that p75 plays a role in discriminating among neurotrophins, thereby tuning the specificity of neurotrophin binding.

In addition to its potential role in neurons, p75 may serve a variety of functions in non-neuronal cells. The observation that p75 is expressed on Schwann cells in regenerating peripheral nerves (Johnson et al., 1988) and in target fields prior to and during their innervation (Wyatt et al., 1990) has led to the suggestion that it may function to concentrate and present the neurotrophins to the high-affinity receptors on the growing axons. Moreover, in vitro experiments using antisense oligonucleotides to prevent translation of p75 mRNA suggest that p75 plays a role in kidney morphogenesis (Sariola et al., 1991) and recent results suggest a role for p75 in neuronal patterning through its influence on the migration of developing Schwann cells (Anton and Matthew, 1992).

To elucidate the role of p75 in neural development, we previously generated mutant mice carrying a null mutation in the p75 locus by gene-targeting in embryonic stem cells (Lee et al., 1992). Mutant mice are viable and exhibit markedly decreased cutaneous sensory innervation by calcitonin-gene related peptide and substance P immunoreactive fibers in the periphery, which is associated with decreased heat sensitivity and the development of skin ulcerations in the extremities (Lee et al., 1992). Mutant animals also display tissue-specific deficits in the sympathetic innervation (Lee et al., unpublished results). For example, while the sympathetic innervation from SCG to iris is normal, the pineal gland lacks sympathetic innervation. Furthermore, sympathetic innervation of the sweat glands is selectively depleted in some footpads.

The phenotype of the peripheral nervous system in p75 mutant animals could result from deficits in the neurons, Schwann cells, or target tissues. As a first step in elucidating the role of p75 in the neurotrophin signal transduction in neurons, we examined neurotrophin sensitivity in p75-deficient DRG and SCG neurons. Our results show that p75-deficient DRG and SCG neurons display a decreased sensitivity to NGF during the period of naturally occurring cell death. Because the concentration of NGF in the target field is limiting, decreased sensitivity would result in excessive loss of DRG and SCG neurons in vivo. This finding indicates that p75 plays a role in mediating neurotrophin function in some neurons and can partially explain the phenotype of p75 mutant mice.

MATERIALS AND METHODS

Animals

Embryos were obtained from timed-pregnant mice and staged by the criteria of Theiler (1972; day of the presence of vaginal plug = E0). In most cases, embryos homozygous for the p75 mutation were obtained from crosses between outbred mutant animals on a mixed background of inbred strains 129 and Balb/c (for details, see Lee et al., 1992). Control embryos were obtained from crosses from inbred Balb/c mice. To determine whether the response of neurons was dependent on p75 gene dosage, mice heterozygous for the p75 mutation were intercrossed. The genotypes of individual embryos were determined by Southern blotting analysis (Lee et al., 1992).

Neuron cultures

DRGs dissected from embryos were collected in L15 medium (Gibco) and were incubated for 7 minutes at 37°C with 0.05% trypsin (Worthington) in calcium- and magnesium-free Hanks balanced salt solution (HBSS). Trypsin was neutralized by adding 10 ml of F12 medium containing 10% heat-inactivated horse serum (Gibco). After washing once with the same medium, the ganglia were gently triturated with a fire-polished Pasteur pipette to obtain a suspension of single cells and were plated at a density of 200–400 neurons per 35 mm dish (Nunc), which had been coated overnight at room temperature with poly-DL-ornithine (Sigma; 0.5 mg/ml, in 0.15 M borate buffer, pH 8.3) and subsequently with laminin (BRL; 20 µg/ml) for 4 hours at 37°C. The neurons were cultured in F14 medium (Imperial Laboratories Ltd., UK) supplemented with 2 mM glutamine, 0.35% bovine albumin (Pathocyte-4, ICN), 60 ng/ml progesterone, 16 µg/ml putrescine, 400 ng/ml l-thyroxine, 38 ng/ml sodium selenite, 340 ng/ml tri-iodo-thyronine, 60 µg/ml penicillin and 100 µg/ml streptomycin.

For postnatal SCG neurons, ganglia were dissected and cleaned free of connective tissue using tungsten needles and were incubated for 30 minutes with a solution of collagenase (Worthington; 1 mg/ml) and dispase (Boehringer-Mannheim; 5 mg/ml) in HBSS. The ganglia were washed twice with HBSS and were incubated with 0.25% of trypsin (Gibco) in HBSS for 30 minutes at 37°C. The ganglia were triturated and cultured as described above for DRG neurons.

In each experiment, triplicate cultures were established for all conditions. 6-12 hours after plating, the number of attached neurons within a 12×12 mm square in the centre of each dish was counted to determine the initial number of neurons for the experiment. After 48 hours incubation, the percentage of neurons surviving in the presence or absence of different neurotrophins was determined by counting the neurons that were phase-bright and neurite-bearing in the same 12×12 mm area in each dish. The results are expressed as a percentage of initial number of attached neurons.

RESULTS

p75-deficient DRG neurons have a decreased sensitivity to NGF

To examine whether p75-deficient DRG neurons have an altered response to NGF during the mid to late embryonic period when naturally occurring neuronal death occurs, dose-response experiments were performed on cultures of DRG neurons isolated from E15, E17, and E19 embryos. Virtually no neurons survived in the absence of neurotrophins after 48 hours in culture. The NGF dose-response of wild-type DRG neurons varied with age (as seen in Fig. 1; A. M. Davies, unpublished results). The concentration of NGF required to promote the survival of half of the wild-type neurons increased from approximately 10 pg/ml at E15 to 100 pg/ml at E19 (Fig. 1). When p75-deficient DRG neurons from the same stage were examined, no difference in response at saturating concentrations of NGF was observed (2 or 10 ng/ml). However, at sub-saturating concentrations of
NGF, p75-deficient DRG neurons were two- to three-fold less sensitive to NGF compared with wild-type neurons (Fig. 1A-C). The most pronounced shift in the NGF dose-response was seen in E15 cultures; the half-maximal NGF concentration was 10 pg/ml for wild-type neurons and 30 pg/ml for p75-deficient neurons (P<0.05, t-test).

To determine if p75 gene dosage affects the response of DRG neurons to NGF, E14 DRG neurons were isolated from individual embryos obtained from a cross between mice heterozygous for the p75 mutation and were subjected to the survival assay with NGF. The genotypes of individual embryos were determined and were matched with the survival results. The NGF dose responses of trigeminal neurons from wild-type (filled circle), heterozygous (filled triangle) and homozygous (open circle) embryos are shown. Cultures were set up in triplicate for the neurons from each embryo, and the combined mean and standard error of the mean are plotted.

### Neurotrophin specificity is unchanged in p75-deficient DRG neurons

Experiments using avian DRG neurons have demonstrated that distinct populations of neurons depend on different neurotrophins for survival in vitro (Davies et al., 1986; Hory-Lee et al., 1993). In situ hybridization studies have revealed that while individual trks are expressed in subsets of DRG neurons, most, if not all, DRG neurons express p75 (Yan and Johnson, 1988). Recently, Rodriguez-Tébar et al. (1992) have suggested that p75 plays a role in discriminating among neurotrophins because different neurotrophins, although having similar affinity, exhibit significantly different dissociation rates with p75. For this reason we examined whether neurotrophin specificity was altered in p75-deficient DRG neurons. E15 DRG neurons were isolated and cultured in the presence of NGF, BDNF, NT-3, or NT4/5. As illustrated in Fig. 3, saturating concentrations of all four neurotrophins (NGF, BDNF, NT-3, or NT4/5) promoted the survival of a similar percentage of DRG neurons. The results demonstrate that p75 plays a role in the survival response of embryonic DRG neurons to NGF.
neurons from both control and p75-deficient neurons. Thus, p75 does not play a role in neurotrophin discrimination in E15 DRG neurons at saturating concentrations.

p75-deficient postnatal SCG neurons have a decreased sensitivity to NGF

SCG neurons undergo naturally occurring cell death during the first week of postnatal life with the peak from P3 to P7 (Wright et al., 1983). Most, if not all, SCG neurons express p75 and trkA (Yan and Johnson, 1988; Ernfors et al., 1992; Schecterson and Bothwell, 1992) and depend on NGF for survival in vitro (Levi-Montalcini, 1987). Because sympathetic innervation by SCG in some target tissues was decreased, we examined the response of p75-deficient SCG neurons to different neurotrophins. For this, P3 or P4 SCG neurons from wild-type and p75 mutant animals were cultured in parallel in the presence of NGF. Like p75-deficient embryonic DRG neurons, postnatal SCG neurons from mutant animals exhibited a small but significantly decreased sensitivity to NGF (Fig. 4). Interpolation of the survival curves indicated that the half-maximal concentration was significantly greater for p75-deficient SCG neurons (150 pg/ml) than for wild-type neurons (50 pg/ml) (P<0.05, t-test). This finding shows that p75 also plays a role in enhancing the NGF sensitivity in postnatal SCG neurons in contrast to SCG neurons isolated from embryos (Davies et al., 1993).

p75-deficient SCG neurons are more sensitive to NT-3

While most SCG neurons are dependent on NGF, NT-3 has been shown to elicit neurite-outgrowth and support the survival of small percentage of SCG neurons (Dechant et al., 1993). Interestingly, low levels of trkC, the cognate receptor for NT-3, were detected by in situ hybridization in all SCG neurons although it was not known whether the signals represented the full-length or truncated receptor (Ernfors et al., 1992). We therefore studied if responsiveness to NT-3 was altered in p75-deficient SCG neurons. Consistent with previous findings (Dechant et al., 1993), the results in Fig. 5 show that a very small percentage of SCG neurons was supported by NT-3 at 2 or 10 ng/ml. This is in marked contrast to the survival of the majority of SCG neurons with 10 ng/ml NGF (compare Figs 4 and 5). Although the survival of SCG neurons increased with higher concentrations of NT-3, it had not reached a maximum with NT-3 concentrations as high as 250 ng/ml. Surprisingly, p75-deficient SCG neurons were more responsive to NT-3 than control neurons at concentrations of NT-3 higher than 10 ng/ml (Fig. 5). These results indicate that the p75 mutation results in an increased sensitivity of postnatal SCG neurons at high concentrations of NT-3.

DISCUSSION

p75 mutant mice were used to investigate the role of p75 in the
survival of sensory and sympathetic neurons. Our results show that p75-deficient embryonic DRG and postnatal SCG neurons are 2- to 3-fold less sensitive to sub-saturating concentrations of NGF than wild-type neurons. This may help explain the phenotype of p75 mutant mice. Because the concentration of NGF is limiting in the target fields of sensory and sympathetic neurons during development, the decreased sensitivity of p75-deficient sensory and sympathetic neurons to NGF may lead to excessive loss of neurons in DRG and SCG in vivo. Consistent with this possibility, p75 mutant mice have substantially reduced numbers of CGRP and SP immunoreactive nerve fibres in the periphery (Lee et al., 1992) and it has been shown that SP-positive and CGRP-positive DRG neurons respond to NGF (Kessler and Black, 1980; Otten et al., 1980; Lindsay and Harmar, 1989). p75 mutant mice also have decreased sympathetic innervation in sweat glands and the pineal gland (Lee, unpublished results) and it is known that the in vivo survival of SCG neurons is dependent on a supply of NGF in the postnatal period (Levi-Montalcini, 1987).

Previously, we have shown that embryonic p75-deficient trigeminal ganglion sensory neurons, like DRG neurons, are less sensitive to NGF than wild-type neurons (Davies et al., 1993). Trigeminal ganglion neurons are born in the mid-embryonic period and undergo naturally occurring cell death between E12 and E18 in vivo (Davies and Lumsden, 1984). Although the period of naturally occurring cell death for DRG neurons in mouse has not been documented, these neurons are also born in the mid-embryonic period (Sims and Vaughn, 1979) and likely undergo naturally occurring cell death during the same period as trigeminal neurons. Therefore, the shift in the survival response of p75-deficient DRG neurons to higher NGF concentrations is observed at the time of naturally occurring cell death. This shift may be correlated with the expression of p75 and trkA mRNAs, both of which increase at the onset of NGF responsiveness at E12 (Wyatt and Davies, 1993) and are maintained at similar levels throughout embryonic development. In contrast to our present finding that postnatal p75-deficient SCG neurons are less sensitive to NGF than aged-matched wild-type neurons, our previous results showed that embryonic SCG neurons from p75 and wild-type mice respond similarly to NGF (Davies et al., 1993). This age-related difference in the response of SCG neurons from p75-deficient and wild-type mice may be related to the late development of sympathetic neurons and developmental changes in the levels of p75 and trkA mRNAs in these neurons. SCG neurons are born before birth but undergo naturally occurring cell death only during the first postnatal week (Whitt et al., 1983). The level of trkA mRNA in embryonic SCG neurons begins to increase with acquisition of NGF responsiveness at E14, but in contrast to sensory neurons the level of p75 mRNA remains low until the late fetal period before any substantial increase is observed; at E17 the ratio of trkA mRNA to p75 mRNA is over six to one and by P3 this ratio has decreased to less than two (S. Wyatt and A. M. Davies, unpublished data). Thus, the decreased NGF responsiveness of sensory and sympathetic neurons in p75-deficient mice is observed only during the stage of development when the level of p75 is normally elevated, i.e., in sensory neurons during the mid to late embryonic period and in SCG neurons during the postnatal period.

TrkA is essential for the NGF signal transduction pathway which is probably initiated by ligand-induced homodimerization (Jing et al., 1992). The hallmark of this pathway is phosphorylation of a succession of proteins that includes phospholipase C-g1 and MAP-2 kinase (Gomez and Cohen, 1991; Loeb et al., 1992; Miyasaka et al., 1991; Vetter et al., 1991; Ohmichi et al., 1992; Thomas et al., 1992; Wood et al., 1992). Activation of this pathway results in the expression of a variety of gene products that are required for neuronal survival and morphological differentiation. Because p75 does not contain a tyrosine kinase domain, its role in neurotrophin signal transduction is not clear. Our results, however, indicate that p75 is involved in NGF sensitivity in DRG and SCG neurons. The mechanism(s) by which p75 increases NGF sensitivity in these neurons is not clear. There are at least three possibilities that are not mutually exclusive. First, it is known that NGF-sensitive DRG and SCG neurons possess a 10-fold higher number of low-affinity receptors than high-affinity receptors. The low-affinity receptors may function to present the neurotrophin more efficiently to the high-affinity receptor trkA, thereby increasing NGF sensitivity. Second, p75 may interact directly with trkA for the formation of high-affinity binding and the signalling response as suggested by Hempstead et al. (1991). Third, p75 may interact with other proteins. For example, when PC12 cells are treated with NGF, two protein kinases, p130 and 6-thioguanine-sensitive protein kinase N (PKN), become phosphorylated and associated with p75 (Ohmichi et al., 1991; Volonte et al., 1993). Therefore, it is possible that the activation of p130 and PKN and possibly the activation of other unidentified proteins may increase the efficiency of the trk-mediated intracellular protein phosphorylation cascade.

It was surprising that p75-deficient SCG neurons were more sensitive to higher concentrations of NT-3 than wild-type neurons, raising the possibility that p75 might modulate the response of neurons to neurotrophins other than NGF. Developing sympathetic neurons are known to express both trkA (Enfors et al., 1992; Schecterson and Bothwell, 1992) and trkC (Enfors et al., 1992). Although trkC is the cognate receptor for NT-3 (Lamballe et al., 1991), NT-3 is capable of eliciting a mitogenic response in fibroblast cells expressing trkA in the absence of p75 (Ip et al., 1993). Thus, it is possible that the effect of high concentrations of NT-3 on the survival of SCG neurons may be mediated by either trkA or trkC. Perhaps p75 may contribute to the specificity of NT-3 binding and, in its absence, high levels of NT-3 may promiscuously interact with trkA and enhance the survival of SCG neurons. Consistent with this interpretation, NT-3 displays a rather limited effect on PC12 cells (which express high levels of p75) as compared to that on trkA-expressing fibroblast cells. Moreover, expression of a truncated p75 receptor in PC12 cells results in decreased expression of wild-type p75 and increased neurite out-growth in response to NT-3 (Benedetti et al., 1993). Alternatively, in the absence of p75, NT-3 may activate trkC more efficiently. It should be noted, however, that the concentration of NT-3 used in our experiment is probably above physiological levels (above nanomolar ranges; 50 ng/ml equal to 1.9x10^{-9} M). Because the concentration of NT-3 in the target field is unknown but likely to be quite low, this result may have no physiological relevance and may be of pharmacological interest only. At present, we do not understand why DRG neurons do not exhibit a similar change in their response to
Several lines of evidence demonstrate that cell type as well as the physiological context of cells are important for elucidating neurotrophin signal transduction pathway. For example, 100-fold higher concentrations of NT-3 are required to elicit cellular responses in trkB-expressing PC12 cells than fibroblasts expressing the same receptor (Berkemeier et al., 1991; Cordon-Cardo et al., 1991; Squinto et al., 1991; Ip et al., 1993). In addition to cell type, the developmental stage of neurons may influence their response to neurotrophins. The availability of p75 mutant mice provides an opportunity to examine the responses of different types of neurons to neurotrophins at different stages of development, as illustrated in this report. Generating mice carrying mutations in individual trks and intercrossing these mutant mice with p75 mutants will undoubtedly further facilitate our understanding of the neurotrophin signaling pathway in an appropriate physiological context.

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REFERENCES


Kessler, J. A. and Black, I. B. (1980). Nerve growth factor stimulates the
brain-derived neurotrophic factor to the nerve growth factor receptor. Neuron 4, 487-492.


