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# Multiple effects of artemin on sympathetic neurone generation, survival and growth

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## SUMMARY

**To define the role of artemin in sympathetic neurone development, we have studied the effect of artemin on the generation, survival and growth of sympathetic neurones in low-density dissociated cultures of mouse cervical and thoracic paravertebral sympathetic ganglia at stages throughout embryonic and postnatal development. Artemin promoted the proliferation of sympathetic neuroblasts and increased the generation of new neurones in cultures established from E12 to E14 ganglia. Artemin also exerted a transient survival-promoting action on newly generated neurones during these early stages of development. Between E16 and P8, artemin exerted no effect on survival, but by P12, as sympathetic neurones begin to acquire neurotrophic factor independent survival, artemin once again enhanced survival, and by P20 it promoted survival as effectively as nerve growth factor (NGF). During this late period of development, artemin also enhanced the growth of neurites from cultured neurones more effectively than NGF. Confirming the physiological relevance of the mitogenic action of artemin on cultured neuroblasts, there was a marked reduction in the rate of neuroblast proliferation in the sympathetic ganglia of mice lacking the GFRα3 subunit of the artemin receptor. These results indicate that artemin exerts several distinct effects on the generation, survival and growth of sympathetic neurones at different stages of development.**

Key words: Artemin, Sympathetic neurones, Mouse, Neurotrophins

## INTRODUCTION

Artemin (Baloh et al., 1998) is the most recently identified member of the glial cell line-derived neurotrophic factor (GDNF) family of neurotrophic factors, which includes neurturin (Kotzbauer et al., 1996) and persephin (Milbrandt et al., 1998) in addition to the founding member GDNF (Lin et al., 1993). These secreted proteins promote the survival of various kinds of neurones of the peripheral nervous system (Suter-Crazzolara and Unsicker, 1994; Buj-Bello et al., 1995; Trupp et al., 1995; Kotzbauer et al., 1996; Wright and Snider, 1996; Molliver et al., 1997; Baloh et al., 1998; Heuckeroth et al., 1998; Forgie et al., 1999; Baudet et al., 2000) and central nervous system (Lin et al., 1993; Henderson et al., 1994; Arenas et al., 1995; Ha et al., 1996; Williams et al., 1996; Milbrandt et al., 1998) and play a role in the development of several other organs (Moore et al., 1996; Pichel et al., 1996; Sanchez et al., 1996; Cacalano et al., 1998; Enomoto et al., 1998). Moreover, some populations of neurones require different members of this family of proteins at different stages of their development; for example, parasympathetic neurones switch responsiveness from GDNF to neurturin during development (Forgie et al., 1999; Enomoto et al., 2000).

Members of the GDNF family use multicomponent receptors that consist of a common receptor tyrosine kinase signalling component Ret (Durbec et al., 1996; Trupp et al., 1996; Vega et al., 1996; Worby et al., 1996), plus one of a family of GPI-linked receptors (GFRα1 to 4) that confers ligand specificity. Studies of ligand binding, Ret phosphorylation and the responses of cells expressing these receptors have indicated that Ret/GFRα1 is the preferred receptor for GDNF (Klein et al., 1997; Jing et al., 1996; Treanor et al., 1996), Ret/GFRα2 is the preferred receptor for neurturin (Buj-Bello et al., 1997; Baloh et al., 1997; Creedon et al., 1997; Jing et al., 1997; Sanicola et al., 1997; Suvanto et al., 1997; Widenfalk et al., 1997; Trupp et al., 1998), Ret/GFRα3 is the preferred receptor for artemin (Baloh et al., 1998) and Ret/GFRα4 is the receptor for persephin (Enokido et al., 1998).

The defective formation and maintenance of the sympathetic nervous system in mice with a disrupted *GFRα3* (*Gfra3* – Mouse Genome Informatics) gene (Nishino et al., 1999) prompted our present study to clarify the role of artemin in sympathetic neurone development. GFRα3-deficient mice survive to adulthood but exhibit the characteristic features of defective cranial sympathetic function. Although the number of cells in the superior cervical ganglion (SCG) of *GFRα3*$^{-/-}$ embryos is apparently normal at E11.5 and continues to increase between E12.5 and birth, the number is only 60 to 80% of the number in wild-type SCG during this period of

embryonic development. The SCG is also displaced more caudally in *GFRα3*$^{-/-}$ embryos at E12.5 and later stages, possibly owing to failure of SCG precursors to complete the last step in their rostral migration from lower cervical levels (Nishino et al., 1999). It is unclear, however, whether this defect in precursor cell migration in GFRα3-deficient embryos is the cause of the reduced number of cells in the early SCG or whether artemin-GFRα3 signalling plays a role in sympathetic neuroblast proliferation or differentiation. By birth, the sympathetic innervation of several tissues is defective in *GFRα3*$^{-/-}$ mice, and during the postnatal period there is progressive death of neurones in the SCG so that by P60 fewer than 5% of the neurones remain (Nishino et al., 1999). It is unclear, however, whether this loss of SCG neurones is because sympathetic neurones depend on artemin for survival or whether the neurones fail to obtain other target-derived neurotrophic factors because artemin/GFRα3 signalling plays a key role in establishing sympathetic innervation. To shed light on these issues, we have examined the effects of artemin on sympathetic neurone generation, survival and growth in dissociated sympathetic ganglion cultures established over a broad range of ages from E12 to adulthood. We show that artemin promotes neuroblast proliferation and neurone generation, and that it enhances neurite growth and sustains sympathetic neurone survival at different stages of development.

## MATERIALS AND METHODS

### Neurone culture

Low-density dissociated cultures were established separately from the SCG and from the stellate ganglion together with other ganglia of the thoracic sympathetic chain (SG) of CD1 mouse embryos at 12, 13, 14, 15 and 16 days gestation (E12 to E16), and at birth and postnatal days 4, 8, 12, 16, 20, 35 and 60 (P0 to P60). The dissected ganglia were trypsinised (0.05% trypsin for 15 minutes at 37°C) and dissociated by trituration. P12 and older ganglia were treated with collagenase (0.2% collagenase for 30 minutes at 4°C followed by 20 minutes at 37°C) before trypsinisation. The neurones were grown in defined, serum-free medium on a poly-ornithine/laminin substratum in 35 mm diameter tissue culture petri dishes (Davies et al., 1993). Purified recombinant human artemin or function-blocking mouse monoclonal anti-GFRα3 antibodies were added to the cultures at the time of plating.

To obtain a simple estimate of the number of neurones surviving in these cultures under different experimental conditions, the number of attached neurones within a 12×12 mm grid in the centre of each dish was initially counted 6 hours after plating and was counted again at time intervals thereafter. The number of neurones present in the grid at these later times is expressed as a percentage of the initial count at 6 hours. In each experiment, triplicate cultures were set up for all conditions.

Because neurones are generated from proliferating progenitor cells in cultures of early SCG and SG, the number of neurones surviving in these cultures at intervals following plating is influenced not only by the length of time individual differentiated neurones survive, but also by the rate at which neurones are generated from their progenitors. To quantify both of these parameters in the same experiment, we followed the survival of the neurones that comprised an initial cohort identified shortly after plating, monitored the generation of new neurones at intervals and followed the survival of these newly generated neurones. In these 'cumulative cohort experiments', the initial cohort was identified within a 12×12 mm grid in the centre of 60 mm culture dishes 6 hours after plating. The survival of these neurones was monitored at 6 hourly intervals and is expressed as a percentage of the starting number of neurones in the initial cohort. In addition to following the survival of neurones in the initial cohort, the generation of new neurones in the same grid was monitored at each time point. This established new cohorts of neurones that were generated between 6 and 12 hours, 12 and 18 hours, 18 and 24 hours, 24 and 30 hours, and 30 and 36 hours. The survival of neurones in each of these newly identified cohorts was subsequently monitored at 6 hourly intervals after their identification. The number of neurones in these cohorts is expressed as a percentage of the number of neurones in the initial cohort identified 6 hours after plating. The results of each experiment are plotted in stacking bar charts.

### Neuroblast proliferation in vitro

Neuroblast proliferation was measured in vitro by determining the number of neuroblasts that incorporated bromodeoxyuridine (BrdU) into their nuclei using immunocytochemistry. Cells were plated in 24-well multiwell plates (Costar), BrdU was added at various times after plating the cells and the cultures were incubated for a further period to permit incorporation of BrdU into S-phase cell nuclei. The cells were then fixed in methanol (–20°C for 15 minutes) and were stained for nuclear BrdU incorporation using an anti-BrdU monoclonal antibody (Sigma) diluted 1:500 in phosphate-buffered saline (PBS) for 48 hours at 4°C. The cells were then labelled using biotinylated secondary antibody (1:200), avidin and biotinylated horseradish peroxidase macromolecular complex (Vectastain ABC Kit, Vector Labs). The substrate used for the reaction was diaminobenzidine tetrachloride (DAB substrate kit, Vector Labs). The number of BrdU-positive cells is expressed as a percentage of total cell number.

### Generation of *GFRα3*$^{-/-}$ mice

A BAC clone containing the *GFRα3* gene was isolated and used to construct a targeting vector. A pGK1-neo cassette flanked 5′ by a 1.6 kb PCR fragment located approximately 1.5 kb upstream of the initiation ATG and 3′ by a 5.3 kb *Eco*RI-*Xho*I fragment immediately downstream of exon 1 was used to delete a 2 kb fragment containing the first exon of GFRα3. Linearised DNA (20 μg) was used to electroporate $1\times10^7$ ES R1 cells. G418/Gancyclovir resistant clones were screened for homologous recombination by Southern analysis. Genomic DNA was digested with *Hin*dIII and hybridised with a 1.5 kb EcoRI-PstI fragment located upstream of the 1.6 kb short arm. Homologous recombination was detected at a frequency of 1/100 clones. Three independent targeted clones were used to generate chimeric animals by injection into C57BL/6 blastocysts. Male chimeras were bred with C57BL/6 females, and heterozygous offspring were interbred to generate *GFRα3*$^{-/-}$ mice. These mice were back-crossed into a CD1 background (the same in which all other experiments were carried out).

### Neuroblast proliferation in GFRα3-deficient embryos

Embryos were obtained from overnight matings of *GFRα3*$^{+/-}$ mice. Pregnant females were killed by cervical dislocation after 14 days gestation, and the genotypes of the embryos were determined by a PCR-based technique using DNA isolated from embryonic bodies. The heads were fixed for 30 minutes in Carnoy's fluid (60% ethanol, 30% chloroform and 10% glacial acetic acid). After dehydration through a graded alcohol series, the tissue was paraffin wax embedded. Serial sections of the heads in the region of the SCG were cut at 8 μm and were mounted onto poly-lysine-coated slides (BDH) or Gold Seal Ultrastick Slides (Erie Scientific).

To monitor neuroblast proliferation, the sections were stained for the presence of the proliferating cell nuclear antigen (PCNA). Because expression of PCNA is not restricted to dividing neuronal cells, the sections were double stained for βIII tubulin to identify all neuroblasts and neurones in the SCG. The sections were cleared in xylene and rehydrated before quenching in 3% hydrogen peroxide in methanol

for 20 minutes. Nonspecific antibody binding was blocked in 10% horse serum, 0.5% Triton X-100 in PBS before incubation with anti-PCNA monoclonal antibody (Sigma) diluted 1:1000 in blocking buffer for 1 hour at room temperature. The cells were then labelled using biotinylated secondary antibody (1:200), avidin and biotinylated horseradish peroxidase macromolecular complex (Vectastain ABC Kit, Vector Labs). The substrate used for the reaction was 1mg/ml diaminobenzidine tetrachloride (FastDAB, Sigma). The sections were then incubated with mouse anti-βIII tubulin antibody (Promega) diluted 1:5000 in blocking buffer overnight at 4°C. The cells were then labelled using biotinylated secondary antibody (1:200), avidin, biotinylated horseradish peroxidase macromolecular complex (Vectastain ABC Kit, Vector Labs) and visualised using a VIP substrate kit (Vector Labs) which produced an intense purple reaction product.

The total number of βIII tubulin-positive cells and the number of βIII tubulin-positive cells that were also PCNA positive were estimated using a digital stereology system that uses a combination of the optical dissector and volume fraction/Cavalieri methods (Kinetics Imaging).

## RESULTS

### Artemin enhances the generation and survival of early sympathetic neurones

We began investigating the role of artemin in sympathetic neurone development by studying its effects on neuronal survival in dissociated cultures established from the SCG and SG at closely staged intervals throughout embryonic development. Preliminary analysis showed that in cultures established throughout the period of neurogenesis from E12 to E15, there were more neurones in artemin-supplemented cultures than in control cultures. Fig. 1 illustrates the dose-dependent nature of this effect of artemin and shows that the most effective concentration for increasing neurone number was 10 ng/ml, which was used in all subsequent experiments.

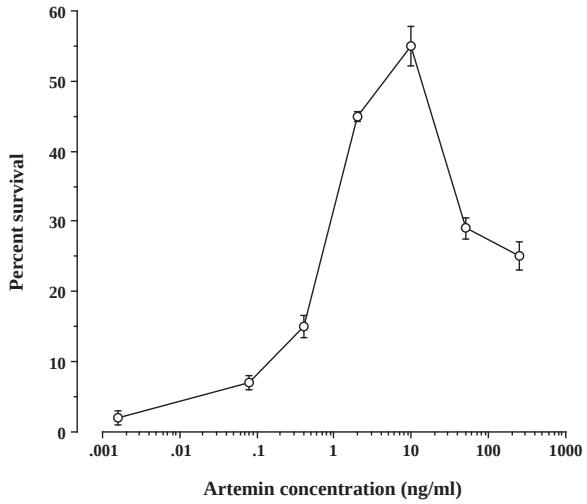


**Fig. 1.** Graph of the number of sympathetic neurones present in E14 dissociated SCG cultures after 48 hours incubation with a range of concentrations of artemin expressed as a percentage of the number of neurones counted 6 hours after plating. The means and standard errors (minus survival in control cultures) of data obtained from three petri dishes for each condition are shown. Similar results were obtained in two separate sets of cultures.

In cultures established after the period of neurogenesis (from E16 to birth), no increase in neurone number was observed in the presence of artemin.

Because sympathetic neurones are generated from proliferating neuroblasts in dissociated cultures of early sympathetic ganglia (Rohrer and Thoenen, 1987), it is possible that artemin could increase the number of surviving neurones in these cultures by either increasing the generation of neurones from neuroblasts or by enhancing neuronal survival. To distinguish between these two alternatives, we set up cumulative cohort experiments. This involved identifying all of the neurones within a grid in the centre of each culture dish 6 hours after plating and following the fate of every neurone in this initial cohort at 6 hourly intervals (i.e. whether it was alive or dead). Differences in rate at which the neurones die between control and artemin-supplemented cultures would indicate whether artemin enhances the survival of differentiated neurones. In addition, all new neurones that were generated in the grid during successive 6 hourly intervals were identified and their fate was likewise followed over time. These latter data would indicate whether artemin influences the generation of neurones from neuroblasts. Fig. 2 shows the results of typical cumulative cohort experiments set up at daily intervals from E12 to E16. In all cultures, the survival of the initial cohort of neurones was enhanced in the presence of artemin compared with control cultures. This survival-promoting effect of artemin was most marked in E12 to E14 cultures, it was less evident in E15 cultures and was negligible in E16 cultures. These results indicate that artemin has a direct survival-enhancing action on neurones present in the early sympathetic chain, but that this effect is transient and not maintained beyond E16.

The cumulative cohort experiments showed that new neurones are generated in early sympathetic chain cultures. Most new neurones were generated in E12 cultures and the number of new neurones decreased with age, becoming negligible by E16 (Fig. 2). Expressed as a percentage of the size of the initial 6 hour cohorts, the total number of new neurones generated in control cultures between 6 and 36 hours decreased from 70% in E12 cultures to 3% in E16 cultures (Fig. 3). At all ages, more neurones were generated in artemin-supplemented cultures, the increase was most pronounced in E12, E13 and E14 cultures, when neurogenesis is greatest in control cultures. In E12 to E14 cultures there were 60% more new neurones generated in the presence of artemin (Fig. 3). This increase in neurogenesis in the presence of artemin was evident during each 6 hourly interval in culture (Fig. 2). The results of these cumulative cohort experiments suggest that artemin increases the proliferation of sympathetic neuroblasts.

To provide an additional measure of neuroblast proliferation, we studied BrdU incorporation in dissociated cultures established from E12 and E13 SCG and SC, stages when neurogenesis is highest. Artemin increased the number of BrdU-positive neurones in E12 and E13 cultures by 76% and 55%, respectively (Fig. 3). These results clearly demonstrate that artemin promotes sympathetic neuroblast proliferation in vitro.

### Reduced neuroblast proliferation in GFRα3-deficient embryos

To ascertain whether the effects of artemin on neuroblast proliferation observed in vitro are physiologically relevant, we

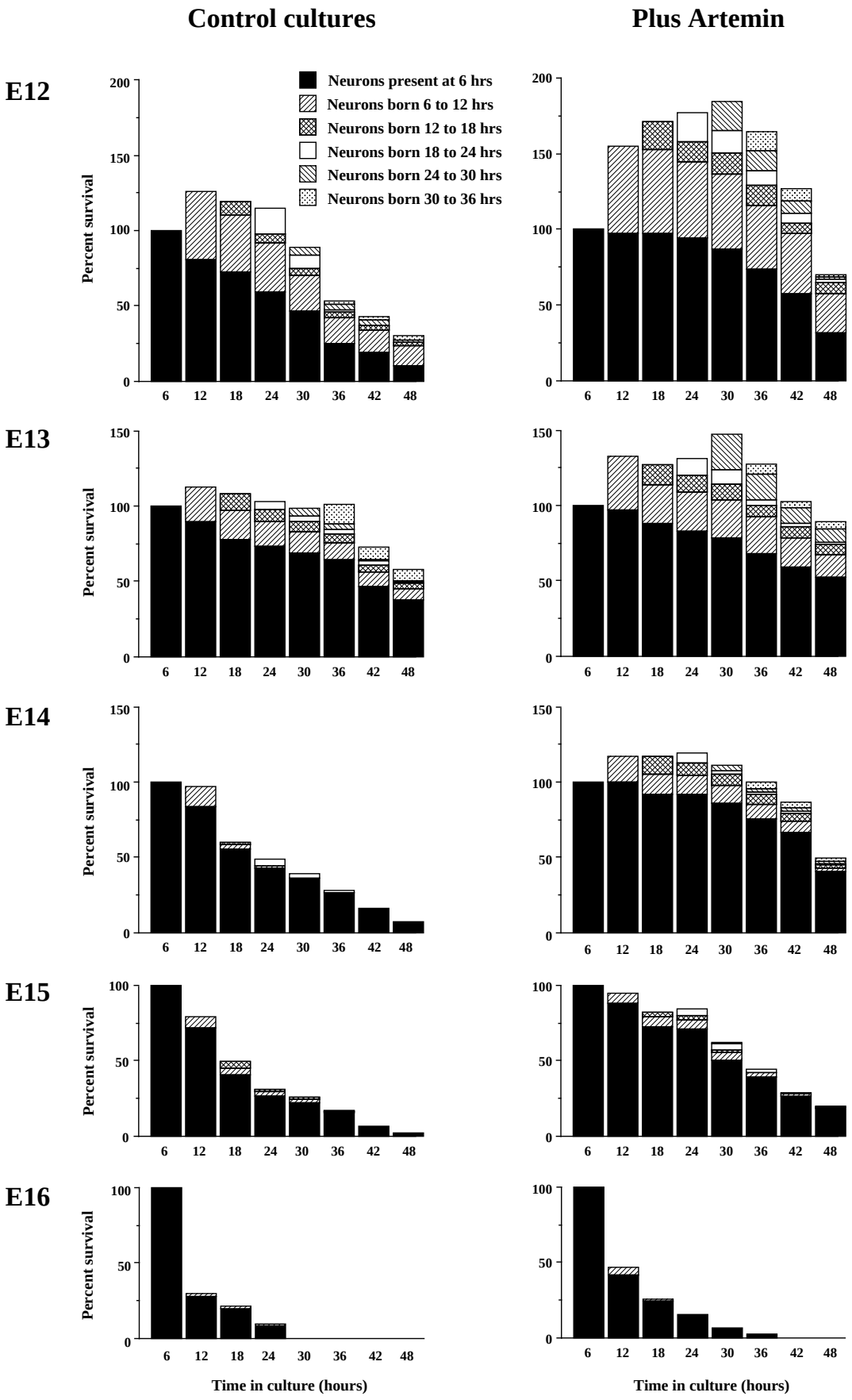


**Fig. 2.** Stacking bar charts of representative cumulative cohort experiments set up from the SCG and SG of E12 to E16 embryos (SCG at E12, E14 and E16, and SG at E13 and E15). The cells were grown in either defined medium alone (control cultures) or medium supplemented with 10 ng/ml artemin. An initial cohort comprising all the neurones in a 12×12 mm grid in the centre of each dish was identified 6 hours after plating. The number of surviving neurones in this cohort was monitored at 6 hourly intervals and is expressed as a percentage of the initial cohort at 6 hours (black bars). New neurones that were generated in the grid between 6 and 12 hours, 12 and 18 hours, 18 and 24 hours, 24 and 30 hours, 30 and 36 hours are expressed as a percentage of the initial cohort, and their survival was likewise subsequently monitored at 6 hourly intervals after their appearance (patterned and white bars).

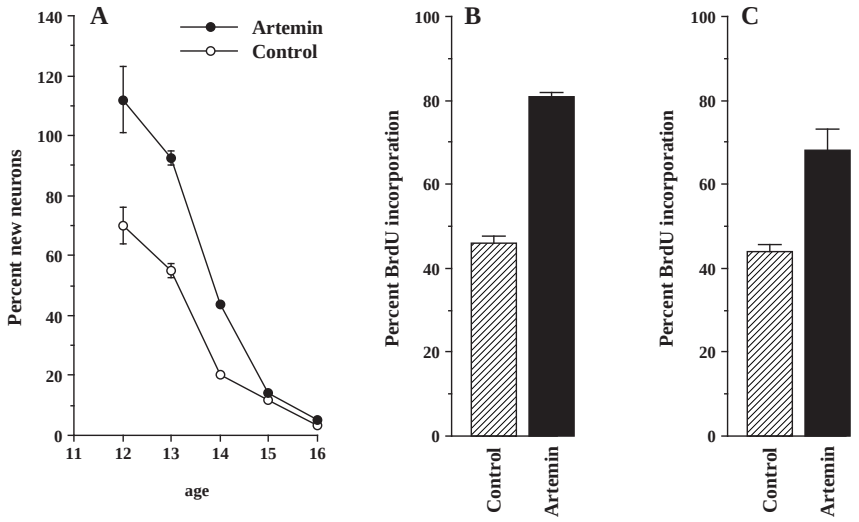


**Fig. 3.** (A) Graph of the number of new neurones generated between 6 and 36 hours after plating in dissociated cultures of sympathetic ganglia of E12 to E16 embryos in defined medium alone (control) or medium supplemented with 10 ng/ml artemin. Bar charts of the number of BrdU-labelled neurones in dissociated E12 SCG (B) and E13 (C) stellate ganglion cultures grown either in defined medium alone (control) or medium supplemented with 10 ng/ml artemin. In E12 cultures, BrdU was added 2 hours after plating and the cultures were fixed and stained 16 hours after plating. In E13 cultures, BrdU was added 24 hours after plating and the cultures were fixed and stained 12 hours later. The means and standard errors are shown ($n$=3-6 for each condition).

generated GFRα3-deficient mice by gene targeting and compared neuroblast proliferation in the E14 SCG of wild-type and *GFRα3*$^{-/-}$ embryos. A targeting vector where the first exon containing the signal sequence was replaced by a PGK1-neo cassette was transfected into embryonic stem cells (Fig. 4). Gene targeting was detected in 1/100 ES colonies and three clones were selected for microinjection into blastocysts. Chimeric mice with appropriate germline transmission were back-crossed into a CD1 background.

For the experimental studies, *GFRα3*$^{+/-}$ mice were crossed and pregnant females were killed after 14 days gestation. Wild-type and *GFRα3*$^{-/-}$ embryos were fixed, paraffin wax embedded and serially sectioned through the SCG. Proliferating cells were detected by staining these sections with an antibody against proliferating cell nuclear antigen (PCNA), a protein that is expressed at high levels in the nuclei of S-phase cells (Kurki et al., 1986). Although the great majority of cells in E14 SCG are neuroblasts or post-mitotic neurones, to ensure that only PCNA-positive neuroblasts were counted, we also stained these sections for β-III tubulin, which labels neuroblasts and post-mitotic neurones. Confirming the results of Nishino and colleagues (Nishino et al., 1999), we found that the SCG of *GFRα3*$^{-/-}$ embryos were smaller and more caudally located than the SCG of wild-type embryos. This difference in the overall size of the SCG of *GFRα3*$^{-/-}$ embryos was associated with a reduction in the number of β-III tubulin-positive cells compared with wild-type embryos in the same litter (Fig. 5). Importantly, the proportion of β-III tubulin-positive cells that were PCNA-positive in the SCG of *GFRα3*$^{-/-}$ embryos was half that observed in wild-type embryos. These results indicate that a smaller proportion of neuroblasts undergo mitosis in the SCG of GFRα3-deficient embryos compared with wild-type

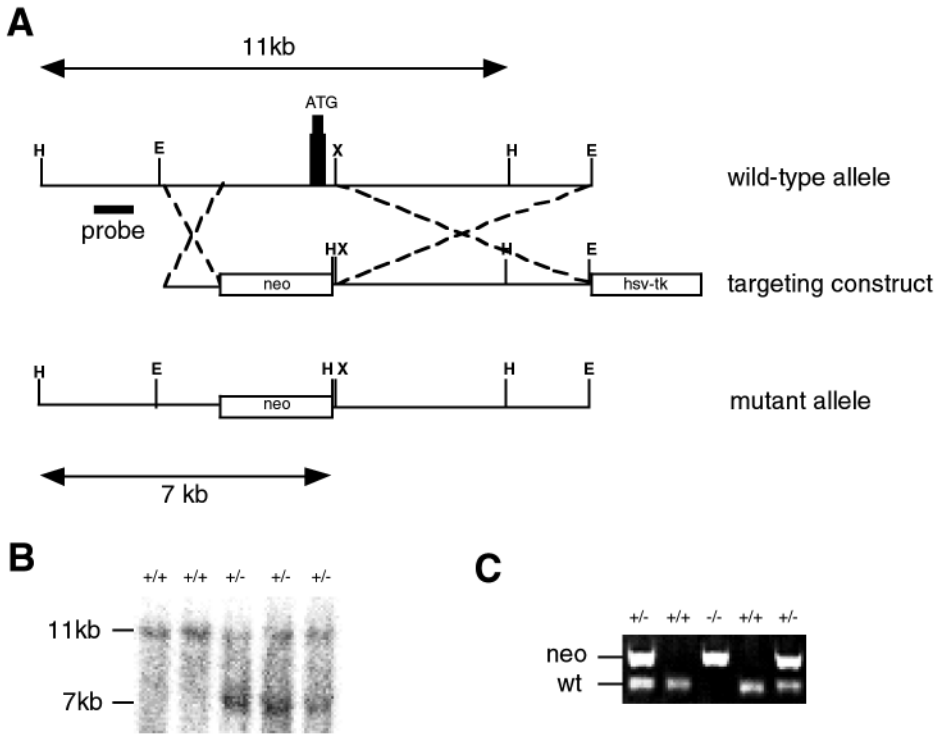


**Fig. 4.** Generation of GFRα3 knockout mice by homologous recombination. (A) Gene targeting vector. A Neo cassette replaced a fragment containing exon one, deleting the start codon and the signal sequence of GFRα3. H, *Hin*dIII; E, *Eco*RI; X, *Xho*I. (B) Southern blot screening of targeted ES clones. Genomic DNA was digested with *Hin*dIII. The probe, upstream of the short arm (shown in A), detected the 11 kb wild type and 7 kb mutant fragments. (C) PCR genotyping of tail DNA prepared from offspring of intercrossing heterozygous mice. Tail DNA was amplified with a primer set specific for the Neo gene, which detects the mutant allele, and a primer set that hybridises within the sequence that is deleted in the mutant and therefore detects the wild-type allele.

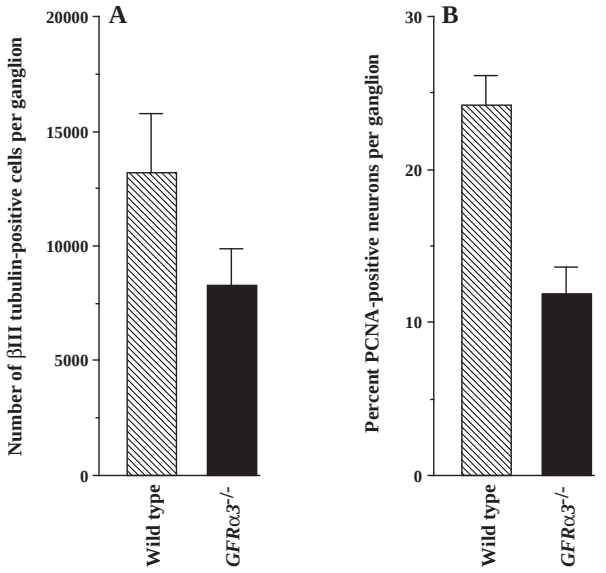


**Fig. 5.** Bar charts of (A) the total number of βIII-tubulin-positive and (B) the percent of βIII-tubulin-positive cells that are PCNA positive in E14 wild-type and *GRFα3*$^{-/-}$ embryos. The means and standard errors are shown (data derived from six wild-type and five *GRFα3*$^{-/-}$ embryos).

embryos, suggesting that the GFRα3 ligand artemin promotes sympathetic neuroblast proliferation in vivo.

## Artemin has a transient survival effect on mature SCG neurones

To investigate whether artemin affects the development of sympathetic neurones after birth and in the adult, we investigated the effects of artemin on SCG neurones in low-density dissociated cultures established at intervals throughout the postnatal period and into adulthood. From birth to P8, neurones in artemin-supplemented cultures died as rapidly as neurones in control cultures, so that by 48 hours incubation all neurones grown in control and artemin-supplemented cultures had died. By contrast, nerve growth factor (NGF) promoted the survival of the majority of these neurones and survival was neither enhanced nor reduced by the concomitant presence of artemin (Fig. 6).

As sympathetic neurones began to lose their survival dependence on NGF with age, they started to exhibit a clear survival response to artemin. In P12 cultures, the neurones died much more slowly in control cultures than at earlier postnatal stages, so that by 48 hours incubation, 30% of neurones were still surviving in defined medium alone. In artemin-supplemented cultures at this age, the neurones died more slowly than in control cultures, so that by 48 hours there were 66% neurones still surviving in the presence of artemin. From P16 to P60, the neurones survived increasingly well in the control cultures, so that after 48 hours incubation in P60 cultures, more than half of the neurones were still surviving without neurotrophic factors. Throughout this period, the neurones survived increasingly well with artemin, so that by P20 the neurones survived as well in artemin-supplemented cultures as in cultures containing NGF. There were no significant differences in the number of neurones surviving with NGF alone or NGF plus artemin at all ages studied, indicating the artemin-responsive neurones comprise a subset of NGF-responsive neurones. The changes in the response of neurones to artemin in the postnatal period in relation to their survival under other conditions are summarised in Fig. 7. These results show that sympathetic neurones acquire a late survival response to artemin during the postnatal period.

The appearance of a survival response to artemin just before sympathetic neurones begin to lose dependence on neurotrophic factors for survival raised the possibility that endogenously produced artemin might account for the ability of adult sympathetic neurones to survive without added neurotrophic factors. Neurotrophic factor autocrine loops have previously been described for BDNF in neurotrophic factor independent embryonic and adult DRG neurones (Wright et al., 1992; Acheson et al., 1995) and for HGF in sympathetic neuroblasts (Maina et al., 1998). To investigate the possibility that artemin released into the culture medium of adult sympathetic neurones is responsible for sustaining the survival of these neurones in the absence of added neurotrophic factors, we used a function-blocking anti-GFRα3 monoclonal antibody. In initial experiments, we confirmed that this antibody inhibited the survival enhancing effects of artemin, and by using different concentrations of this antibody we determined that 100 ng/ml of this antibody was sufficient to block completely the survival-enhancing effects of 10 ng/ml artemin in cultures of E14 SCG neurones (data not shown). Fig. 8 shows that this concentration of antibody also completely inhibited the survival-enhancing effect of artemin on P60 SCG neurones but did not affect the response of the neurones to NGF. The anti-GFRα3 antibody did not reduce the survival of P60 SCG neurones grown without added neurotrophic factors in the culture medium, indicating that the survival of these adult neurones in the absence of added neurotrophic factors is not dependent on the artemin synthesised in these cultures acting on GFRα3 receptors. We also investigated whether endogenously produced artemin plays a role in sustaining sympathetic neurones during the early phase of neurotrophic factor independence. In E14 cultures the anti-GFRα3 antibody did not reduce neuronal survival in the absence of added neurotrophic factors, indicating that endogenously produced artemin released from cells in these cultures does not play a role in sustaining the generation or survival of early sympathetic neurones.

## Artemin enhances the growth of SCG neurone arbours

To investigate the potential effects of artemin on sympathetic neurite growth, it was essential to compare the same subset of neurones grown with and without artemin. Because more neurones survive in artemin-supplemented cultures than in control cultures, even at P60 (when many neurones survive without added neurotrophic factors), simply comparing neurite growth in artemin-supplemented and control cultures would not distinguish between direct neurite-growth promoting effects of artemin and neurite growth that was secondary to the enhanced survival and well being of the neurones in the presence of artemin. However, our finding that the number of neurones surviving in cultures containing NGF plus artemin was no greater than the number surviving in cultures containing NGF alone enabled us to investigate the effects of artemin on

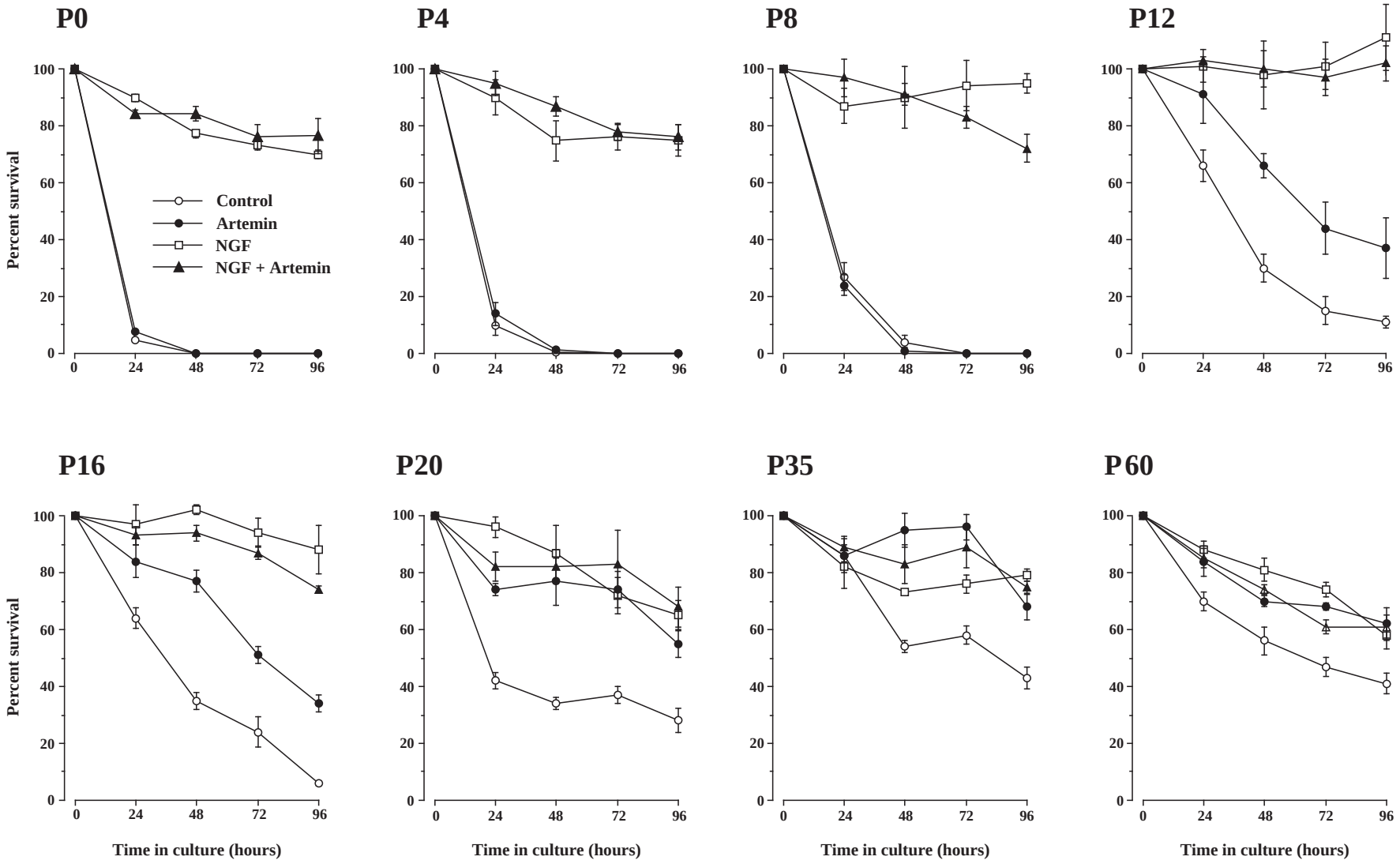


**Fig. 6.** Graphs of the percent survival of P0 to P60 SCG neurones grown in either defined medium alone (control) or in medium supplemented with 10 ng/ml artemin, 10 ng/ml NGF or artemin plus NGF. The number of neurones surviving 24, 48, 72 and 92 hours after plating is expressed as a percentage of the number of neurones counted 3 to 6 hours after plating. The means and standard errors are shown (*n*=6-9 for each condition).

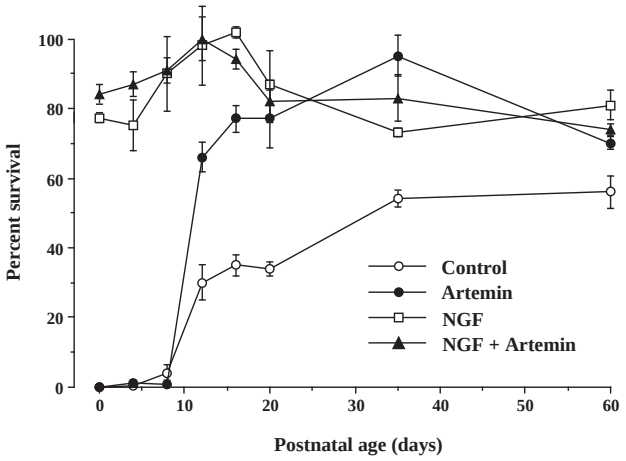


**Fig. 7.** Graph summarising the developmental changes in the percent survival of SCG neurones grown in either defined medium alone (control) or in medium supplemented with 10 ng/ml artemin, 10 ng/ml NGF or artemin plus NGF in cultures established from P0 to P60 mice. The means and standard errors are shown (*n*=6-9 for each condition).

neurite growth independently of survival, and to determine whether artemin was able to enhance neurite growth beyond that observed with NGF alone. Neurones were grown in medium containing NGF in the presence or absence of artemin and the total length of the neurite arbours was estimated. Cultures were set up at stages throughout sympathetic neurone development. At stages between E14 and P3, there were no significant differences in the length of the neurite arbours in cultures containing NGF alone or NGF plus artemin (Fig. 9). However, at P20 (after the neurones have acquired a survival responsive to artemin), the length of neurite arbours was 47% longer in the presence of NGF plus artemin compared with NGF alone. Likewise, at P60, artemin promoted a similar increase in neurite length. These results show that in the late postnatal period, artemin increases neurite growth beyond that occurring in the presence of NGF alone.

## DISCUSSION

To clarify the role of artemin in sympathetic neurone development, we have undertaken a detailed in vitro analysis of its effects on sympathetic neurone generation, survival and growth in dissociated cultures established from sympathetic ganglia at stages throughout development, and complimented these in vitro studies with in vivo analysis of neuroblast proliferation in GFRα3-deficient embryos. Our findings have shown that artemin has several distinct effects at different stages of development. First, it enhances sympathetic neuroblast proliferation and increases the generation of sympathetic neurones. Second, it enhances the survival of sympathetic neurones at two distinct stages in their development: initially for a brief period after they have undergone their terminal mitosis in the embryo and once again in the late postnatal period, shortly before they acquire neurotrophic factor-independent survival. Third, it enhances neurite growth from late postnatal and adult sympathetic neurones.

The effect of artemin on enhancing neuroblast proliferation and the generation of post-mitotic sympathetic neurones was revealed by studying BrdU incorporation in dissociated cultures established from early sympathetic ganglia, and by detailed analysis of the generation of new neurones in cumulative cohort experiments set up throughout the phase of neurogenesis in the sympathetic chain. The physiological relevance of these in vitro observations was substantiated by our demonstration that there is a marked reduction in the proportion of sympathetic neuroblasts in the mitotic phase of the cell cycle in embryos deficient in the GFRα3 subunit of the artemin receptor. Although a reduction in the size of the SCG has been reported in GFRα3-deficient embryos, this was attributed to a failure of SCG precursors to complete the last step in their rostral migration from lower cervical levels (Nishino et al., 1999). Although this is the likely reason for the more caudal location of the SCG in

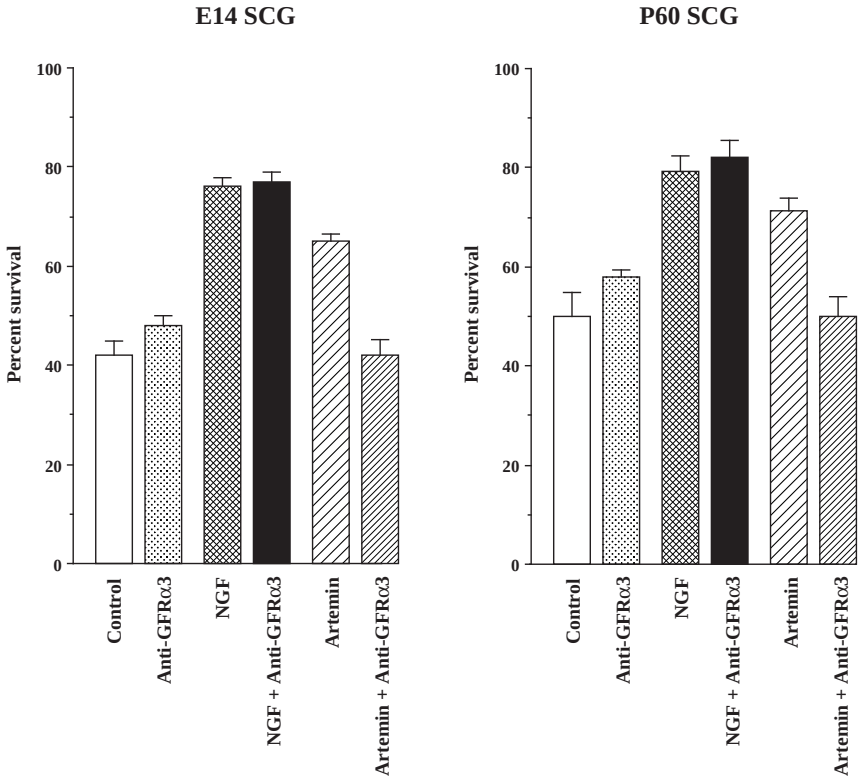


**Fig. 8.** Bar charts of the numbers of surviving neurones in cultures of E14 and P60 SCG grown for 48 and 72 hours, respectively, in either defined medium alone (control) or in medium supplemented with 100 ng/ml anti-GFRα3 antibody, 10 ng/ml NGF, 10 ng/ml artemin, anti-GFRα3 plus NGF or anti-GFRα3 plus artemin. The means and standard errors are shown (*n*=6-9 for each condition).

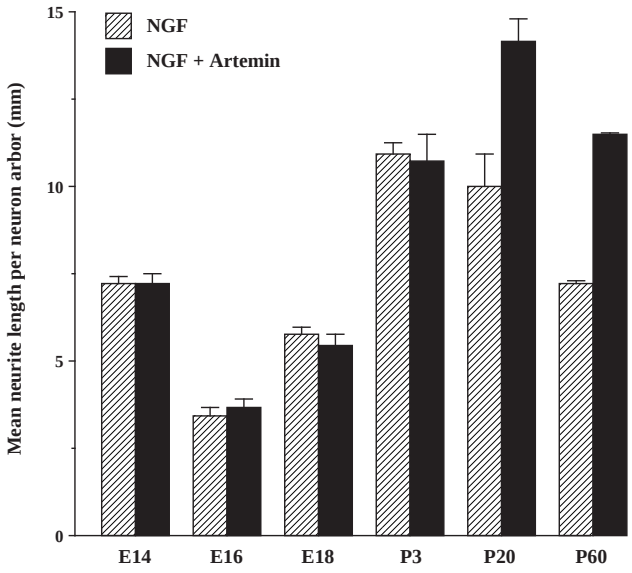


**Fig. 9.** Bar charts of the total length of the neurite arbours growing from SCG neurones in cultures established from E14 to P60 animals after 48 hours incubation with NGF alone or NGF plus artemin (both at 10 ng/ml). The means and standard errors are shown ($n$=4-9 for each condition).

*GFRα3*$^{-/-}$ embryos, and may contribute to the reduced size of this ganglion in these embryos, our findings suggest that a major contributing factor to the decreased size of the SCG is a marked reduction in neuroblast proliferation. A local action of artemin on neuroblast proliferation within the early SCG is consistent with the in vivo expression of artemin and GFRα3. By in situ hybridisation, artemin mRNA is detectable in the SCG and in the region surrounding the SCG precursors, and GFRα3 mRNA is expressed by the majority of cells in the SCG during this early stage in development (Nishino et al., 1999).

What little is known about the signals that potentially regulate sympathetic neuroblast proliferation has been gleaned from in vitro studies of these cells obtained from mammalian and avian embryos. It has been reported that insulin, insulin-like growth factor 1, pituitary adenylate cyclase-activating polypeptide (PACAP), NT3 and retinoic acid promote the proliferation of rat sympathetic neuroblasts in culture (DiCicco-Bloom and Black, 1988; DiCicco-Bloom and Black, 1989; DiCicco-Bloom et al., 1993; Wyatt et al., 1999; DiCicco-Bloom et al., 2000). However, the physiological significance of these in vitro observations is unclear. In the case of NT3, appropriate numbers of sympathetic neurones are generated in NT3-deficient mouse embryos, and enhanced neuronal death occurs only after the phase of neurogenesis is over (Wyatt et al., 1997; Francis et al., 1999). Likewise, the demonstration that CNTF decreases neuroblast proliferation in cultures of embryonic chicken sympathetic chain (Ernsberger et al., 1989) is unclear because in rodents, at least, CNTF synthesis does not commence until after birth (Dobrea et al., 1992). By using complimentary in vitro and in vivo approaches, we have provided the first clear evidence that an identified growth factor plays a physiologically significant role in promoting the proliferation of sympathetic neuroblasts, leading to the increased generation of sympathetic neurones.

Analysis of neuronal survival in cohort experiments established from early sympathetic ganglia clearly shows that artemin sustains the survival of newly generated sympathetic neurones for a brief period after they have undergone their terminal mitosis. This survival effect is no longer evident after E16, and no survival effect of artemin was observed on SCG neurones cultured between this stage and P8. However, by P12, a marked survival response to artemin was observed which was sustained through later ages. The appearance of this late survival response to artemin coincides with the ability of an increasing proportion of sympathetic neurones to survive in culture without added neurotrophic factors. However, the possibility that the production and release of artemin within cultures of mature sympathetic neurones is responsible for the appearance of neurotrophic factor-independent survival is excluded by the lack of effect of function-blocking anti-GFRα3 antibodies on the survival of mature sympathetic neurones.

Previous in vitro studies have reported that artemin promotes the survival of newborn rat sympathetic and sensory neurones (Baloh et al., 1998), and postnatal mouse sensory neurones (Baudet et al., 2000). However, our detailed developmental study has revealed a novel phenomenon that has not previously been described for any other neurotrophic factor, namely, that the same population of neurones exhibits two distinct phases of responsiveness to a factor separated by an extended period during which the factor has no effect on survival. These two phases in the survival-promoting action of artemin on embryonic and late postnatal sympathetic neurones observed in vitro might correspond to local and target-derived actions of artemin, respectively, on sympathetic neurone survival in vivo.

Curiously, although artemin did not promote the survival of cultured SCG neurones between birth and P8 (Figs 6 and 7), these neurones start to die at an abnormally high rate in GFRα3-deficient mice during this period of development (Nishino et al., 1999). It is possible that this abnormal death of SCG neurones may be because absence of artemin/GFRα3 signalling earlier in development might have affected the ability of the neurones to innervate their targets appropriately and to obtain an adequately supply of target-derived NGF and NT3 on which they depend for their survival (Levi-Montalcini and Booker, 1960; Zhou and Rush, 1995). It should be noted that the SCG of *GFRα3*$^{-/-}$ mice forms in an abnormal anatomical location (Nishino et al., 1999) that might affect the subsequent guidance of sympathetic axons to their correct targets.

In addition to enhancing the survival of late postnatal sympathetic neurones, artemin increased neurite growth from these neurones to a greater extent than NGF. The effects of NGF on promoting neurite growth are well documented both in vitro and in vivo (Edwards et al., 1989; Purves et al., 1988; Scott and Davies, 1993; Snider, 1988). Recently, other neurotrophic factors have been shown to synergise with NGF in promoting neurite outgrowth. For example, HGF enhances neurite growth from embryonic sensory and sympathetic neurones in the presence of NGF (Maina et al., 1998; Maina et al., 1997) and enhances neurite growth from postnatal SCG neurones (Yang et al., 1998). It is possible that the effect of artemin on neurite growth observed in vitro may reflect a role for artemin in establishing and maintaining target field innervation in vivo. Indeed, the loss of this effect of artemin in

GFRα3-deficient mice may at least be partly responsible for the observed deficiency of sympathetic axons in several tissues in the postnatal period (Nishino et al., 1999).

In summary, we have defined the roles of artemin at different stages in the development of the sympathetic nervous system by studying its effects on neuroblasts and neurones in culture and by analysing embryos defective in artemin signalling. We have shown that artemin increases sympathetic neuroblast division, and provide the first evidence that a growth factor is a physiologically relevant regulator of the proliferation of these neuroblasts. Artemin subsequently promotes the survival of newly differentiated sympathetic neurones for a brief period after they have undergone their terminal mitosis. Then, uniquely for a neurotrophic factor, artemin promotes the survival of these neurones for a second time several weeks after birth. During this late stage in their development, artemin also enhances neurite growth to a greater extent than NGF. Our results provide a clear illustration of how a single factor can exert a diversity of actions on a population of neurones at different stages in its development and raise interesting questions about the nature of the signalling pathways mediating these different responses.

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