Neurotrophins affect the pattern of DRG neurite growth in a bioassay that presents a choice of CNS and PNS substrates

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SUMMARY

Neurons can be categorized in terms of where their axons project: within the central nervous system, within the peripheral nervous system, or through both central and peripheral environments. Examples of these categories are cerebellar neurons, sympathetic neurons, and dorsal root ganglion (DRG) neurons, respectively. When explants containing one type of neuron were placed between cryosections of neonatal or adult sciatic nerve and neonatal spinal cord, the neurites exhibited a strong preference for the substrates that they would normally encounter in vivo: cerebellar neurites generally extended only on spinal cord, sympathetic neurites on sciatic nerve, and DRG neurites on both.

Neurite growth from DRG neurons has been shown to be stimulated by neurotrophins. To determine whether neurotrophins might also affect the substrate preferences of neurites, DRG were placed between cryosections of neonatal spinal cord and adult sciatic nerve and cultured for 36 to 48 hours in the presence of various neurotrophins. While DRG cultured in NGF-containing media exhibited neurite growth over both spinal cord and sciatic nerve substrates, in the absence of neurotrophins DRG neurites were found almost exclusively on the CNS cryosection. To determine whether these neurotrophin-dependent neurite patterns resulted from the selective survival of subpopulations of DRG neurons with distinct neurite growth characteristics, a type of rescue experiment was performed: DRG cultured in neurotrophin-free medium were fed with NGF-containing medium after 36 hours in vitro and neurite growth examined 24 hours later; most DRG exhibited extensive neurite growth on both peripheral and central nervous system substrates. This experiment demonstrates that the observed neurite growth preferences are due to a nontrophic effect of NGF.

Unlike DRG cultured in NGF, those cultured in the presence of either NT-3 or BDNF often exhibited neurite growth preferences for the spinal cord cryosections; the effect was strongest in cultures containing NT-3. In addition, while a mixture of insulin, transferrin and selenium (ITS) did not by itself have a detectable effect on neurite growth, it dramatically potentiated both BDNF- and NT-3-stimulated neurite growth. However, ITS had no obvious effect on NGF-stimulated neurite growth.

Key words: neurite growth, axon development, neurotrophins, NGF, BDNF, NT-3

INTRODUCTION

Dorsal root ganglia (DRG) contain subpopulations of neurons that differ with respect to their functions, axonal projections, neurotransmitters, cell surface glycoconjugates and carbohydrate-binding proteins, expression of homeobox genes and sensitivity to neurotrophins (Dodd and Jessell, 1986; Regan et al., 1986; Ericson et al., 1992; Klein et al., 1993; Crowley et al., 1994; Ernfors et al., 1994; Farinas et al., 1994; Jones et al., 1994; Klein et al., 1994; Smeyne et al., 1994). While mature DRG and cranial sensory neurons have a pseudounipolar morphology, they have a bipolar morphology during development. These neurons are unique in that they send one axon into peripheral target tissue and the other axon into central nervous tissue. In addition to their divergent axonal trajectories, the two axons of a given DRG neuron differ with respect to their presynaptic morphologies and postsynaptic targets. To date,

however, there is no evidence of differences in membrane-associated molecules that might affect sensory axon growth or guidance. One possibility is that the two axons are identical in this regard and are competent to grow in both central and peripheral nervous system (CNS and PNS) environments. Alternatively, the two axons may develop differences that restrict their capacity to utilize different growth substrates.

Recent gene knockout studies have shown that subpopulations of developing DRG neurons depend on specific neurotrophins for their survival: the small, peptidergic nociceptive DRG neurons are dependent on NGF for survival (Crowley et al., 1994; Smeyne et al., 1994); the large, proprioceptive DRG neurons are dependent on NT-3 (Ernfors et al., 1994; Farinas et al., 1994; Klein et al., 1994); and a subpopulation of DRG neurons, possibly including mechanoreceptors, are dependent on BDNF (Klein et al., 1993; Jones et al., 1994). Recent studies suggest that at least some sensory neurons are sensitive to more

than one neurotrophin (Gaese et al., 1994), particularly during early development (Buj-Bello et al., 1994). Since DRG neurons do not receive afferent innervation, they are likely to be dependent on their CNS (Yip and Johnson, 1984) and PNS targets or perhaps on autocrine sources for neurotrophins. The two axons of a DRG neuron may differ, therefore, with respect to the neurotrophins that they are exposed to.

While much of the research on neurotrophins has focused on their capacity to promote neuronal survival, several studies suggest that neurotrophins also act nontrophically to regulate axon growth and branching (Campenot, 1977; Gundersen and Barrett, 1980; Collins and Dawson, 1983; Lindsay, 1988; Greene et al., 1990; Wright et al., 1992; Lindholm et al., 1993; O'Leary and Daston, 1994; Schnell et al., 1994; Snider, 1994). Indeed, in cultured embryonic DRG, effects on neurite growth precede trophic effects (Wright et al., 1992).

This study examines the preferences that neurites exhibit for CNS or PNS substrates and the effect that neurotrophins have on this substrate-specific growth. To examine these preferences, cryosections of CNS and PNS tissue were placed side by side; neural explants, including DRG, were placed between them and neurite growth on the cryosections examined after 2 or 3 days. Axonal behavior on cryosections tends to mimic that seen in situ (Covault et al., 1987; Sandrock and Matthew, 1987a,b; Tuttle et al., 1989; Tuttle and Matthew, 1991). It has also been demonstrated that allowing neurites to choose between two growth substrates often reveals neurite growth behaviors that are less apparent in a nonchoice paradigm (Bonhoeffer and Huf, 1985; Walter et al., 1987a,b); we have found this to be the case in this cryosection choice bioassay.

MATERIALS AND METHODS

The methods used in the current study have been described in detail elsewhere (Tuttle and Matthew, 1991).

Explant preparation

DRG were removed from embryonic day 7 or 8 White Leghorn chicks – mainly lumbar, upper sacral and lower thoracic. By E7-8, most, if not all, DRG neurons have differentiated (Rohrer et al., 1985); many of the large, early differentiating, ventrolateral neurons have extended axons to their proprioceptive targets, and the period of neuronal death for this population of DRG neurons is underway (Hamburger and Levi-Montalcini, 1949; Hamburger et al., 1981). Superior cervical ganglia (SCG) were removed from decapitated postnatal day (P) 0 or P1 Sprague Dawley rats. DRG and SCG were treated with 0.1% collagenase (Worthington Biochemical Corp.) in DMEM/F12 for 20 minutes in a 37°C incubator, and then rinsed thoroughly. While DRG were explanted whole, SCG were bisected before plating.

Cerebella were removed from decapitated P8 or 9 Sprague Dawley rats, and placed in DMEM/F12 at room temperature. The meninges were removed and 150 μm sagittal sections of cerebellum were cut with a tissue chopper. These sections were washed in DMEM/F12, and if necessary, the long folia were cut into shorter pieces.

Culture media and growth factors

The basic culture medium consisted of DMEM/F12 supplemented with glucose to 0.6%, 5 mM Hepes buffer, 10 μM cytosine arabinoside, 0.5 mM glutamine, 100 Units/ml penicillin, 100 μg/ml streptomycin. This basic culture medium was supplemented with one or more of the following: 5% fetal bovine serum (FBS); ITS (insulin and transferrin at 5 μg/ml and selenium at 5 ng/ml; Boehringer Mannheim); Nutridoma SP (ITS supplemented with BSA; Boehringer

Mannheim); 5-20 ng/ml, 2.5S NGF (Boehringer Mannheim Biochemicals); conditioned medium from untransfected Cos cells (control) or Cos cells that had been transiently transfected with *NT-3* (provided by T. Jessell and M. Tessier-Levigne and used at a 1:100, 1:50, or 1:20 dilution – working concentration of 10, 20, or 50 ng/ml, respectively); and 10-50 ng/ml BDNF (provided by Y.-A. Barde).

Cryosection preparation

Adult rats were anaesthetized with chloral hydrate (350 mg/kg); neonatal rats were decapitated or anaesthetized by hypothermia. Sciatic nerve was quickly dissected from P0 or adult Sprague Dawley rats and the perineurium usually removed. Spinal cord was dissected rapidly from P0 or P1 rats. Tissue was rinsed briefly in cold PBS before freezing onto metal chucks for cryosectioning (Tuttle and Matthew, 1991). 10-20 μm cryosections were dried onto sterile acid-washed 12-mm coverslips that had been treated in one of two ways: either with polylysine, or, to enhance cryosection adhesion, with 3-aminopropyltriethoxysilane and glutaraldehyde (Tuttle and Matthew, 1991); while cerebellar cryosections were coronal, spinal cord and sciatic nerve cryosections were cut along their longitudinal axes. The paired cryosections of CNS and PNS tissue were laid side-by-side on the coverslip, usually with a small gap between them; the order in which the two cryosections were dried down had no apparent effect on the neurite growth patterns observed. Coverslips were then placed in 35-mm Petri dishes and the cryosections rehydrated with culture medium. In some experiments, PNS cryosections were pretreated with NGF. In this case, cryosections were rehydrated with 150 μl of DMEM/F12 containing 15 ng/ml NGF, and left for 1.5 hours at 37°C. Cryosections were then given three, 10 minute washes. Explants were placed between the adjacent cryosections and the medium slowly withdrawn until the surface tension held the explant in place (Tuttle and Matthew, 1991).

Analysis of neurite growth

Cultures were maintained in a 37°C, 2.5% CO_2, humidified incubator for 36-48 hours, unless otherwise noted. Neurite growth was visualized with the vital dye, 5 (and -6) carboxyfluorescein diacetate succinimidyl ester (Tuttle and Matthew, 1991). This dye labels living cells and their processes; the cryosection substrates are, therefore, not labeled with this method. Thus it was possible to distinguish between neurite growth on the cryosection and neurite growth on cells that occasionally migrated onto the cryosection. Cultures were photographed with fluorescein optics. Neurite growth preference data were collected only for DRG that were either centered between the two cryosections or that exhibited a preference for the cryosection that was more distant. To quantitate neurite growth, negatives were digitized with a Microtek ScanMaker (Microtek International, Inc.). The fluorescent image, now represented by pixels, was edited to substract the DRG and surrounding non-neuronal cells, leaving only neurites. *Image* software (NIH, v.1.49) was then used to count the number of pixels as an approximation of total neurite mass.

RESULTS

Neurites of central and peripheral neurons exhibit characteristic growth patterns on cryosection substrates of central and peripheral nervous tissue

Explants of neural tissue placed between longitudinal cryosections of neonatal spinal cord and adult sciatic nerve displayed distinct neurite growth patterns on the paired CNS/PNS substrates. P8 rat cerebellar neurites, which project only within the CNS in vivo, generally did not extend on sciatic nerve cryosections; neurite growth on CNS tissue cryosections was, however, robust (Fig. 1). Cerebellar neurites on the CNS section were more numerous, longer, less fasciculated and
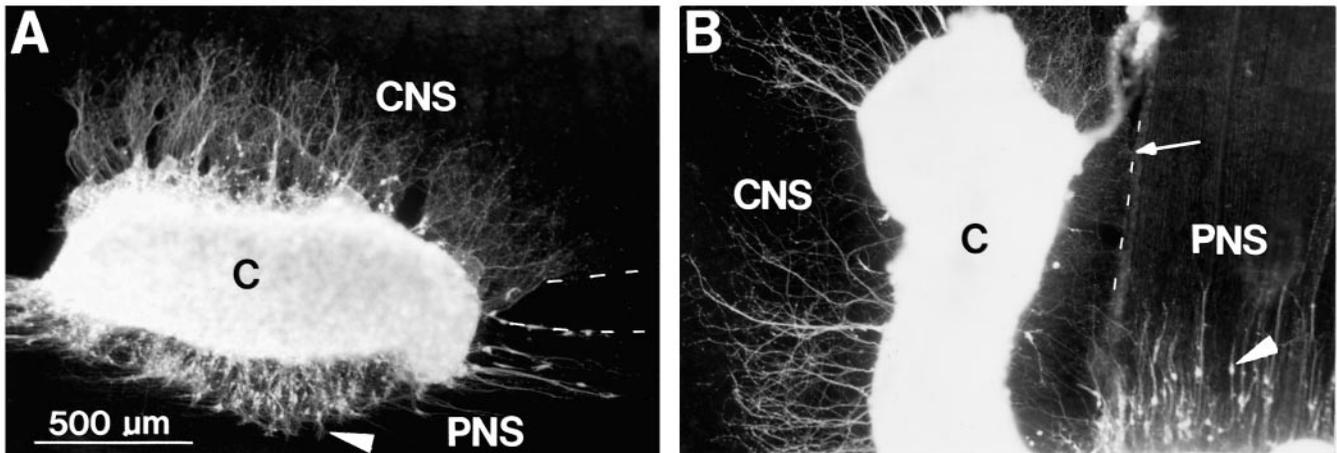
Fig. 1. Cerebellar neurites extend onto a cryosection of CNS but not PNS. P8 rat cerebellar explants were placed between cryosections of P0 lumbosacral rat spinal cord and adult rat sciatic nerve and cultured for 2 days. (A) A cerebellar explant (C) lies between spinal cord (CNS) and sciatic nerve (PNS) cryosections whose boundaries are indicated with dotted lines. There is extensive cerebellar neurite outgrowth on the CNS cryosection, much of it unfasciculated. Neurite growth over the PNS cryosection is generally restricted to cerebellar cells that have emigrated onto the cryosection; neurites that extend beyond these cell islands appear stunted and fasciculated (arrowhead in A). (B) L-shaped cerebellar explant (C) placed over PNS and CNS cryosections that are directly juxtaposed (border indicated with a dashed line). Numerous, long cerebellar neurites extend over the CNS cryosection but seem to stop at the border with PNS (arrow in B). Processes on the PNS cryosection seem largely associated with the numerous bipolar, probably non-neuronal, cells that have migrated out of the explant (arrowhead in B). Basic culture medium (see Materials and methods) was supplemented with Nutridoma SP. Neurites and cells in this and all other figures are visualized with a vital dye that labels all living cells and their processes; the cryosections are not stained with this method.

unlike the growth on the PNS section, were not associated with cells that migrated from the explant. In contrast, neonatal rat SCG, which normally project only within the PNS, exhibited robust neurite growth on the PNS cryosections and, generally poor neurite growth on the CNS cryosections (Fig. 2).
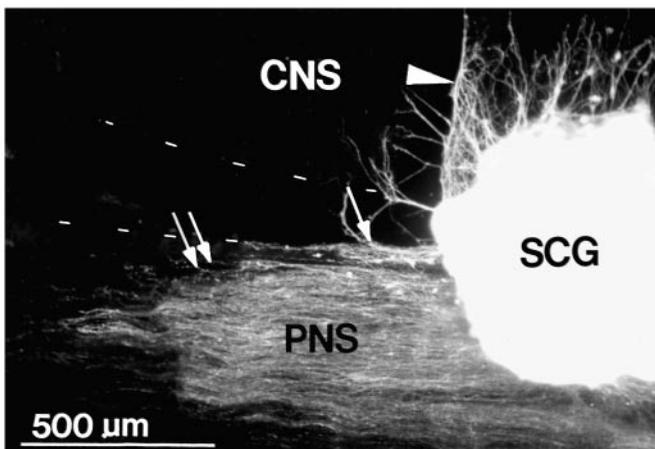

Fig. 2. SCG neurites extend numerous long neurites on PNS, but not on CNS. In the same experiment illustrated in Fig. 1, P1 bisected rat SCG were placed between cryosections of P0 rat spinal cord and adult rat sciatic nerve. SCG neurites on the PNS cryosection are numerous and very long. However, on the CNS cryosections, neurites were short and fasciculated (arrowhead). Neurites extending on the perineurium (single arrow), which in this experiment was not removed, are much shorter than those on the neural parenchyma (double arrow). Cryosection edges are indicated by dashed lines. Basic culture medium was supplemented with Nutridoma SP and 10 ng/ml NGF.

These findings suggest that some CNS and PNS neurites prefer to grow on CNS and PNS substrates, respectively. Therefore, we hypothesized that DRG neurites, which extend in both CNS and PNS environments in vivo, would extend neurites on both CNS and PNS substrates in vitro. To test this, we used chick DRG since substrate preferences have not been found to be species dependent (Godement and Bonhoeffer, 1989), and chick DRG have fewer non-neuronal cells than rodent DRG (non-neuronal cells migrating onto the cryosections complicate the assay). When cultured in NGF-containing medium, embryonic day (E) 7 or E8 chick DRG exhibited exuberant neurite growth on both the sciatic nerve and the neonatal spinal cord cryosections (Fig. 3).

Dissociated, purified P1 mouse DRG neurons or P6 mouse cerebellar neurons plated on cryosections of lightly fixed P1 rat spinal cord or lightly fixed or unfixed P1 rat sciatic nerve exhibited the same neurite growth preferences as did the whole DRG (data not shown). After 24 hours, DRG neurons had extended neurites on both the CNS and PNS cryosections. Cerebellar neurons, however, extended very few neurites on the neonatal sciatic nerve cryosection and these were short and fasciculated; on CNS cryosections and coverslips, cerebellar neurons extended numerous, long neurites. Those on the coverslip grew up to, but not onto the sciatic nerve cryosection. In contrast, DRG neurites extending on the coverslip were able to grow onto the sciatic nerve cryosection. Since the perineurium had been removed from the sciatic nerve, the source of this apparent inhibitory effect is the neural parenchyma.

Neurotrophins alter the distribution of DRG neurites on cryosections of central and peripheral nervous tissue by a nontrophic mechanism

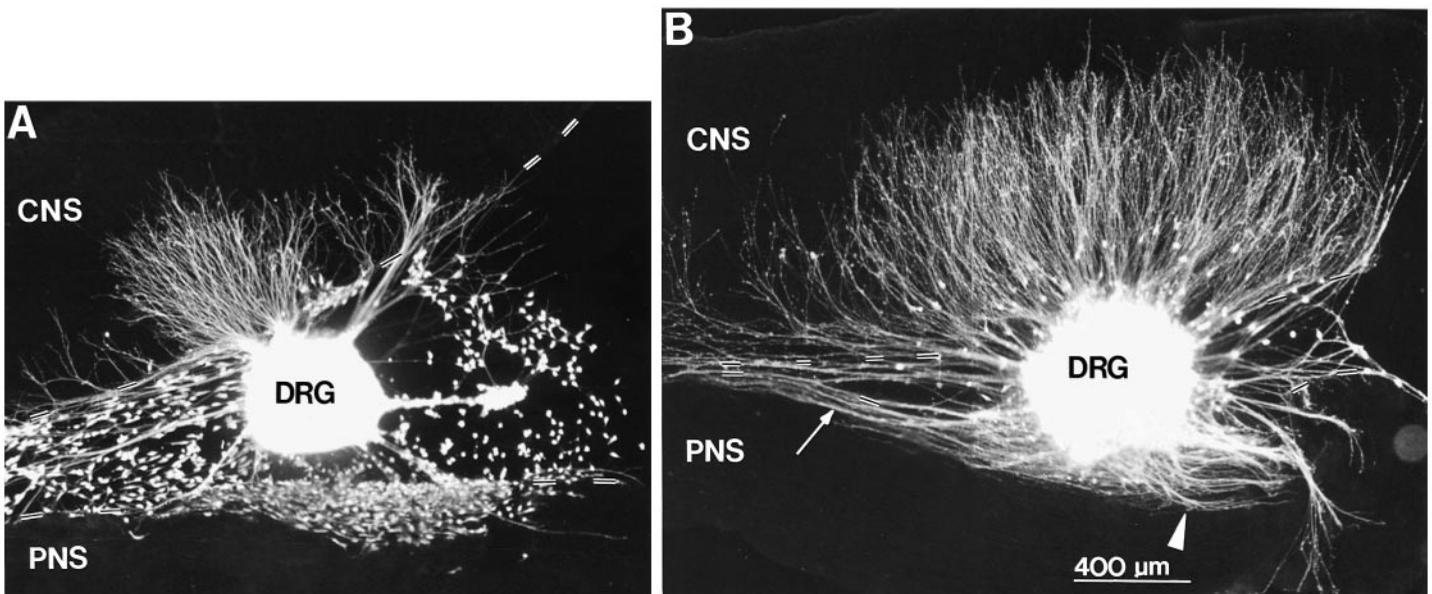DRG cultured in the absence of exogenously added NGF
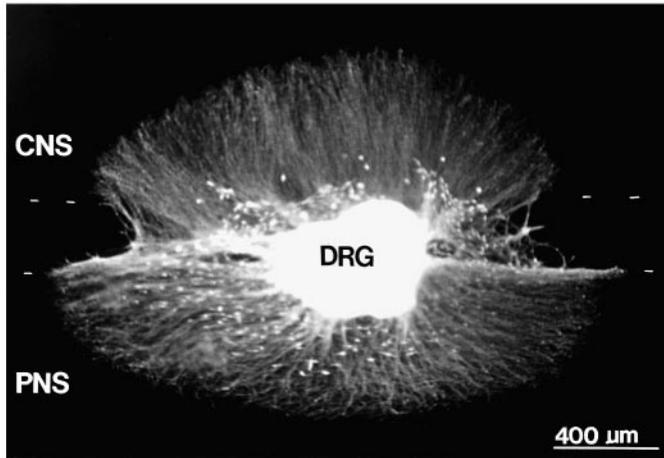
Fig. 3. DRG neurites are able to extend long and numerous processes on both CNS and PNS. E7 chick DRG between cryosections of P0 rat spinal cord and adult rat sciatic nerve exhibit similar levels of neurite growth on the CNS and PNS cryosections. The apposing cryosection edges are indicated with dotted lines. The basic culture medium was supplemented with ITS and 5 ng/ml NGF.

generally exhibited less neurite growth than in similar cultures that contained NGF. Surprisingly, the neurites in these NGF-free cultures exhibited an overwhelming preference for the CNS cryosections (Fig. 4). When DRG were cultured in FBS-containing medium, neurites were much longer, and possibly more numerous; however, without exogenously added NGF, DRG neurites remained restricted to the CNS cryosections (data not shown). The results were not altered by using P0, rather than adult, sciatic nerve (data not shown).

To examine the effects of other neurotrophins on DRG neurite distribution, these experiments were repeated with NT-3 or BDNF (Fig. 5). DRG cultured in medium supplemented with ITS and conditioned medium from untransfected Cos cells extended neurites only onto the CNS substrate (Fig. 5A). In cultures supplemented either with ITS and conditioned medium from Cos cells transfected with *NT-3*, or with ITS and BDNF, neurite growth was much greater. Unlike DRG cultured in NGF, the BDNF and NT-3 stimulated neurite growth often exhibited a preference for the CNS substrate (Fig. 5B,C); the CNS preference was stronger in the NT-3 cultures. The results of the DRG neurite preference experiments are summarized in Fig. 6A. Significantly, DRG neurite growth was never observed only on the PNS substrate.

Neurotrophins could be acting trophically to select for the survival of subpopulations of DRG neurons with distinct growth substrate responses. To test this, DRG were cultured in the choice assay in the absence of exogenously added neu-rotrophins. After 36-48 hours, when DRG exhibit a clear CNS preference (Figs 4, 6A), the cultures were fed with medium containing NGF and examined 24 - 36 hours later. If NGF is having a trophic effect on the DRG, neurites in these cultures should still exhibit a CNS preference. However, the majority of DRG in these cultures exhibited either no preference or a PNS preference (Fig. 6B). In contrast, control cultures fed after 36-48 hours with NGF-free medium, still exhibited a strong preference for the CNS cryosections. In controls in which NGF was present throughout the culture period, DRG neurites displayed either no preference or a preference for the PNS substrate. These experiments demonstrate that the substrate growth patterns exhibited by DRG neurites cultured in the absence or presence of NGF are dictated primarily by non-trophic mechanisms.

It is possible that NGF acts by binding to sciatic nerve

Fig. 4. In the absence of exogenously added NGF, DRG neurites tend to extend only on CNS. E7 chick DRG were placed between cryosections of P0 rat spinal cord and adult rat sciatic. The basic culture medium was supplemented only with ITS. The opposing cryosection edges are indicated with dashed lines. (A) DRG neurites extend well on the CNS and on the cells that are dispersed over the coverslip, but not on the PNS section. (B) While DRG neurite growth is excellent on the CNS section, many fewer neurites are on the PNS section, and these tend to be either short (arrowhead) or in large fascicles (arrow); this is in spite of the fact that part of the DRG is positioned on the PNS section.
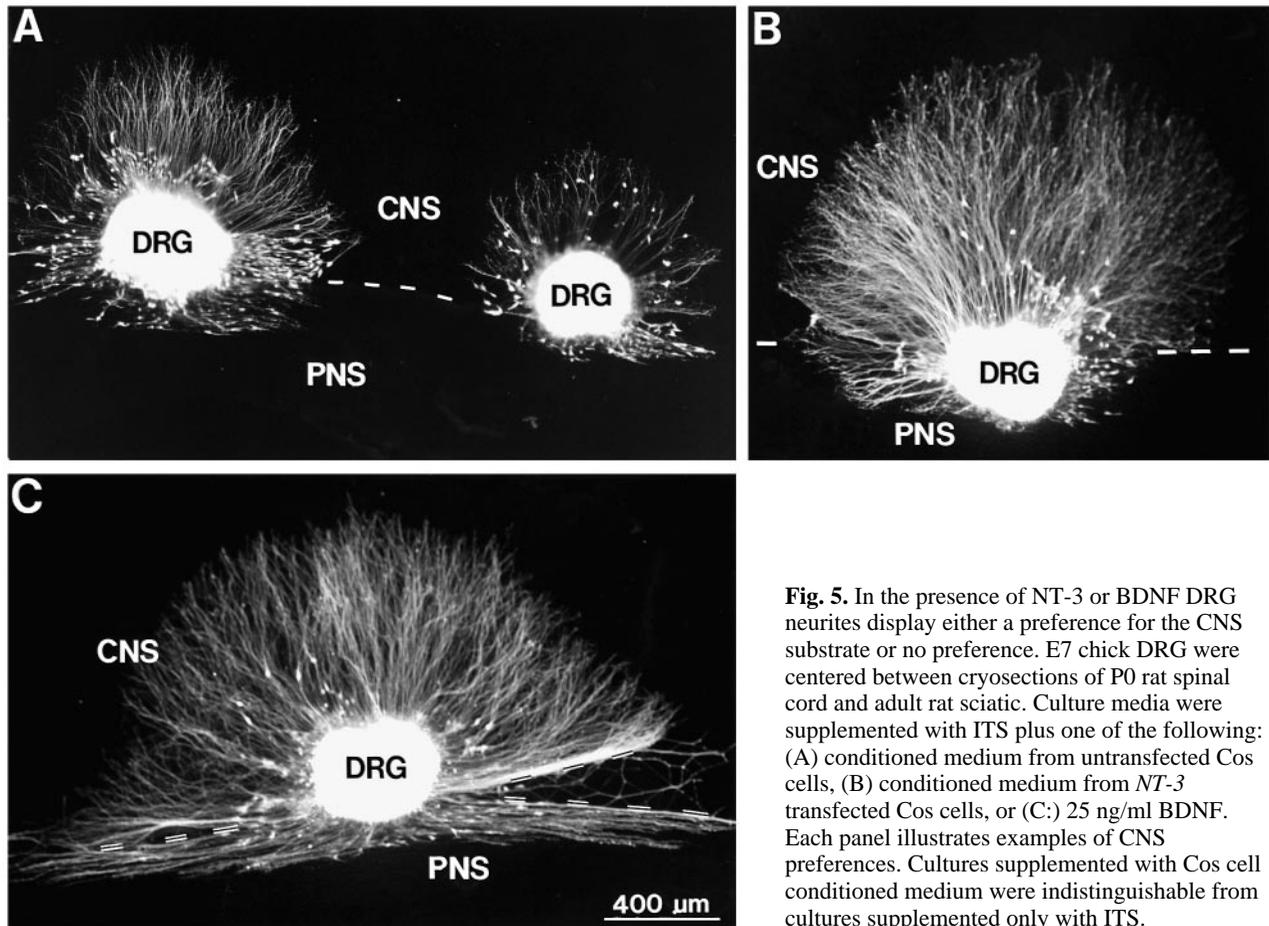
Fig. 5. In the presence of NT-3 or BDNF DRG neurites display either a preference for the CNS substrate or no preference. E7 chick DRG were centered between cryosections of P0 rat spinal cord and adult rat sciatic. Culture media were supplemented with ITS plus one of the following: (A) conditioned medium from untransfected Cos cells, (B) conditioned medium from *NT-3* transfected Cos cells, or (C:) 25 ng/ml BDNF. Each panel illustrates examples of CNS preferences. Cultures supplemented with Cos cell conditioned medium were indistinguishable from cultures supplemented only with ITS.

cryosections, making them more attractive substrates for sensory neurite growth. However, experiments by Sandrock and Matthew (1987b) suggest that NGF does not bind to normal sciatic nerve sections at levels that can be detected by sympathetic neurites. To determine if sensory neurites behave similarly, DRG were placed between two sciatic nerve cryosections pretreated with 15 ng/ml NGF and then washed. We used only sciatic nerve cryosections since the CNS

substrate, having little connective tissue, would not have held up to the washes. DRG neurite growth in these cultures was generally indistinguishable from cultures in which the cryosections had not been treated with NGF (data not shown); that is, DRG neurites tended not to extend onto the PNS substrates. Taken together, the data suggest that NGF acts directly on DRG neurons by a nontrophic mechanism to affect the growth preferences of their neurites.
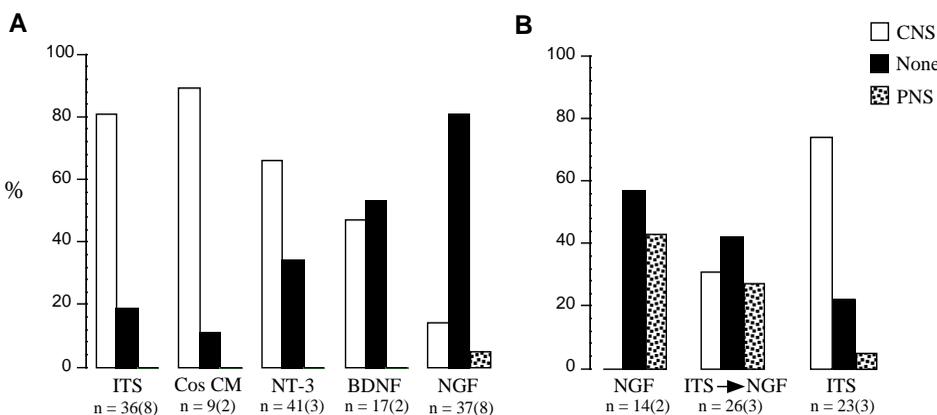
Fig. 6. Neurotrophins effect sensory neurite growth preferences, which, at least in the case of NGF, result from a nontrophic mechanism. (A) Quantitative analysis of all experiments described in Figs 3-5 showing the percentages of DRG whose neurites exhibit a CNS or PNS preference or no preference ('None') after 36-48 hours in vitro in the presence of ITS only, conditioned medium from untransfected Cos cells (Cos CM), conditioned medium from *NT-3* transfected Cos cells, BDNF and NGF. Data for NGF, NT-3, untransfected Cos cell CM and BDNF represent experiments with and without ITS added to the culture medium. For NGF experiments, data for DRG exposed to serum and those cultured in serum-free conditions were also combined. (B) DRG were cultured in the CNS/PNS choice paradigm for 60-70 hours either in the presence or absence of NGF (NGF or ITS, respectively). A third group of DRG were cultured for 36-48 hours in the absence of NGF and then for 24-34 hours more with NGF (ITS → NGF). Numbers in parentheses represent the number of DRG and experiments, respectively.
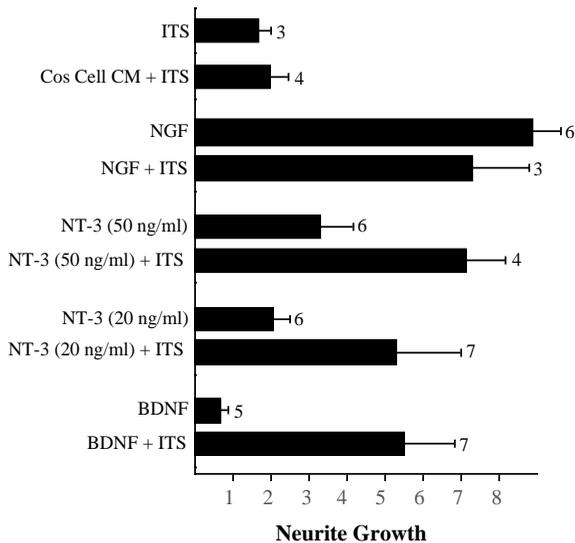
Fig. 7. ITS supplement acts synergistically with NT-3 or BDNF, but not NGF to promote neurite growth. DRG were explanted between cryosections of P1 rat spinal cord and adult rat sciatic nerve and neurite growth on these substrates quantitated as described in Materials and Methods. Mean neurite growth values for a single experiment are represented on the x-axis as a linear scale; s.e.m. bars and the number of explants are also indicated. Basic culture medium (see Materials and methods) was supplemented with ITS alone, or 10 ng/ml NGF, or NT-3 containing Cos cell conditioned medium diluted 1:20 or 1:50 (NT-3 concentration estimated to be 50 or 20 ng/ml, respectively), or 25 ng/ml BDNF each with or without ITS. Conditioned medium from untransfected Cos cells (Cos cell CM; diluted 1:20 or 1:50) was used as a control for the NT-3 producing Cos cells; neurite growth in these control cultures was similar to cultures containing only ITS.

An insulin, transferrin, selenium mixture potentiates the neurite growth-promoting effects of NT-3 and BDNF but not NGF

DRG cultured in the presence of NT-3 or BDNF and no other protein supplement, often exhibited increased amounts of neurite growth compared to cultures supplemented only with an insulin, transferrin, selenium mixture (ITS); in some experiments, however, the differences were minor (Fig. 7). ITS alone, however, consistently had only a weak effect on neurite growth. The amount of neurite growth in response to either BDNF or NT-3, but not NGF, was greatly increased by supplementing cultures with ITS (Fig. 7).

The distribution of DRG neurites grown in NT-3- or BDNF-containing media may be affected by the presence of ITS. While in some NT-3 experiments, the distribution of DRG neurites over a CNS/PNS substrate appeared not to be affected by the presence of ITS, in other experiments the presence of ITS appeared to increase neurite growth on the PNS cryosections. For example, in one experiment 100% ($n = 8$) of the DRG cultured in medium containing both NT-3 and ITS exhibited a preference for CNS that was almost always absolute. However, in another experiment, of DRG grown in NT-3 alone, 82% ($n = 11$) exhibited a CNS preference, while in NT-3 and ITS, only 27% ($n = 11$) exhibited a CNS preference. The effects as well as the number of samples for the BDNF cultures were smaller, and therefore more difficult to assess.

DISCUSSION

CNS/PNS neurite growth preferences

We have shown that rat cerebellar (CNS) and rat sympathetic (PNS) neurites prefer to grow on cryosection substrates from neonatal rat CNS (spinal cord) or neonatal or adult rat PNS (sciatic nerve), respectively. In addition, we have demonstrated that chick DRG neurites, which extend into both CNS and PNS environments in vivo, extend equally well on both CNS and PNS cryosection substrates in vitro. These data suggest that molecules in neonatal CNS inhibit the growth of axons that normally extend only in PNS. Similarly, molecules in neonatal and adult PNS may inhibit the growth of axons that normally extend only within the CNS. Alternatively, CNS and PNS may express different populations of neurite growth-promoters for which neurites exhibit some selectivity (cf. Hankin and Lagenaur, 1994). DRG neurites, which extend in both CNS and PNS, might, therefore, be capable of utilizing both sets of neurite growth-promoters or be indifferent to both presumptive inhibitory signals. Alternatively, the two axons of each DRG neuron may differ with respect to their substrate growth capacities.

It has been shown previously that retinal (CNS) and sympathetic neurites in vitro exhibit a mutual avoidance behavior (Bray et al., 1980; Kapfhammer et al., 1986) due to the contact-mediated collapse of growth cones interacting with heterotypic neurites (Kapfhammer et al., 1986; Kapfhammer and Raper, 1987a,b). Raper and colleagues demonstrated that growth cones of chick PNS neurons (sympathetic, ciliary and DRG) collapse on contact with neurites of chick CNS neurons (retina and diencephalic), and vice-versa (Kapfhammer and Raper, 1987a,b); they have since cloned a growth cone collapsing molecule present in developing chick brain (Luo et al., 1993). In similar experiments, Moorman and Hume (1990) demonstrated that growth cones of a CNS neuron (preganglionic sympathetic) collapse upon contact with neurites of some PNS neurons (DRG) but not others (sympathetic and motoneuron).

Why would DRG neurites be capable of exuberant growth on a CNS cryosection substrate (neonatal spinal cord or postnatal cerebellum) but collapse on contact with some CNS (retinal and diencephalic) neurites? Moorman and Hume (1990) suggest that CNS and PNS axons that do not interact in vivo might be more likely to exhibit repulsive behaviors in vitro; while all DRG axons extend into spinal cord, none interact with retinal or diencephalic neurons. Also in support of this idea, it has been shown that neonatal cortical plate membranes are a good growth substrate for thalamic and cortical neurites, which normally extend in cortex, but are a poor substrate for retinal and cerebellar neurites, which do not (Tuttle et al., 1995). Alternatively, the cryosection contains many more cellular and molecular elements than does an isolated neurite; it is possible that some component(s) in the CNS cryosection modifies or conceals the inhibitory activity revealed so clearly in the growth cone collapse studies.

In apparent conflict with our findings, Aguayo and colleagues have shown that adult rat retinal ganglion cell axons, which normally do not regenerate in vivo, will after 5 months or longer regenerate into grafts of peripheral nerve, suggesting that a PNS environment is not inhibitory for these CNS axons (David and Aguayo, 1981; Vidal-Sanz et al., 1987; Villegas-

Perez et al., 1988). However, unlike the cryosections used in our study, the grafts used by Aguayo and colleagues do not contain axons or myelin but do contain living Schwann cells. In addition, after 5 months, retinal ganglion cell axons grow into the grafts only under particular experimental conditions. It has been demonstrated that both adult and embryonic retinal ganglion cell neurites habituate to neurite growth inhibitors in vitro (Walter et al., 1987a,b, 1990; Cox et al., 1990; Wizenmann et al., 1993); the retinal neurites that extend into the peripheral nerve grafts may be doing the same. Finally, it is worth noting that when one end of the peripheral nerve 'bridge' is inserted into spinal cord, spinal cord cells extend axons into the peripheral nerve graft; however, few cells in the superficial laminae of the dorsal horn extend axons into the peripheral nerve graft (David and Aguayo, 1981). Unlike other parts of the spinal gray matter, the superficial laminae contain neurons that only extend axons in the CNS. Similarly, in agreement with our hypothesis, we have found that explants of dorsal spinal cord tend not to extend neurites onto peripheral nerve cryosections but do extend neurites onto cryosections of neonatal spinal cord; explants of ventral spinal cord, on the other hand, do extend neurites onto peripheral nerve sections (unpublished observations).

The effect of neurotrophins on DRG neurite growth preferences

We report the novel finding that DRG neurite growth preference is affected by neurotrophins: DRG cultured for 2 days in the presence of NGF exhibit neurite growth over both neonatal CNS and adult or neonatal PNS cryosections. However, in the absence of NGF in the culture medium, DRG neurites are generally present only on the CNS substrate. We show that NGF affects neurite growth preferences by a nontrophic mechanism: neurites of DRG cultured for 2 days without neurotrophins, then fed with NGF-containing culture medium and examined 1 day later generally exhibit either no preference or a PNS preference. A nontrophic function for NGF is suggested by previous studies demonstrating that trkA transcripts are present in most, if not all, DRG neurons in E15 mice (Schecterson and Bothwell, 1992) and rats (Ernfors et al., 1993). Our data suggest a minimum of two DRG neurite types: one that can extend on PNS and possibly CNS cryosections, and a second that can only extend on CNS. Expression of the former type, which is insensitive to presumptive inhibitors in PNS cryosections, appears to be NGF-dependent. It is interesting to note that sympathetic neurites, which are also NGF-dependent, prefer to extend only on the sciatic nerve cryosection; cerebellar granule cells are not NGF-dependent, and rarely extend neurites on sciatic nerve cryosections.

DRG cultured in the presence of BDNF and NT-3 extend neurites, at least some of which are also insensitive to the presumptive neurite growth inhibitors in sciatic nerve. NT-3, like NGF, may be acting nontrophically since the transcript for trkC (a receptor for NT-3) is expressed in all DRG neurons in E7 and E8 chicks, the ages used in the current study (Kahane and Kalcheim, 1994); at these ages, the detected transcript presumably codes for the full-length trkC (Escandon et al., 1994). Finally, a nontrophic role for these neurotrophins is supported by previous studies suggesting that NGF, NT-3 and BDNF might regulate functions such as axon growth, branching, and guidance independent of any trophic effect (Campenot, 1977;

Gundersen and Barrett, 1980; Collins and Dawson, 1983; Lindsay, 1988; Greene et al., 1990; Wright et al., 1992; Lindholm et al., 1993; O'Leary and Daston, 1994; Schnell et al., 1994).

In the cryosection bioassay, neurotrophins could exert a nontrophic effect on neurite preference either by acting directly on the DRG neurons, or by binding to the cryosections. Sandrock and Matthew (1987b) have demonstrated that NGF has no perceptible effect on sympathetic neurite growth on cryosections of normal sciatic nerve such as those used in the present study, although it does bind to and promote sympathetic neurite growth over cryosections of the distal stump of transected sciatic nerve. In a similar experiment, we were unable to increase the amount of sensory neurite growth onto sciatic nerve sections by pretreating them with NGF. In addition, the neurite growth patterns for DRG, sympathetic ganglia and cerebellar explants cultured in the presence of NGF were each dramatically different. These data strongly suggest that NGF, and possibly other neurotrophins as well, act directly on DRG neurons to affect neurite growth patterns.

The effect of an insulin, transferrin, selenium mixture on NT-3 and BDNF-stimulated neurite growth

We have demonstrated that when added in combination with either BDNF or NT-3, ITS dramatically potentiates the neurite growth-promoting effects of these neurotrophins. However, ITS did not appear to enhance the amount of NGF-stimulated neurite growth. That this is not due to neurite growth in NGF already being maximal, is suggested by the ability of FBS to greatly enhance NGF-stimulated neurite growth. Previous studies have shown that insulin does not potentiate the neurite growth-promoting effect of NGF on dissociated chick DRG neurons (Burnham et al. 1974; Bothwell, 1982; Recio-Pinto et al., 1986). It is not clear whether insulin is the component in ITS potentiating BDNF and NT-3 stimulated neurite growth in our assay. The fact that neurite growth was often modest in response to NT-3 alone could be due, in part, to the finding that the neurite growth response of embryonic chick DRG ganglia to NT-3, but not NGF or BDNF, varies over the neuraxis with peak responses occurring at midcervical and midlumbar ganglia (Hory-Lee et al., 1993); sacral and thoracic DRG neurons exhibited weak responses to NT-3.

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