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INVESTIGATING THE SYNERGISTIC EFFECT OF COMBINED NEUROTROPHIC FACTOR CONCENTRATION GRADIENTS TO GUIDE AXONAL GROWTH

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Abstract—Neurotrophic factors direct axonal growth toward the target tissue by a concentration gradient, which is mediated through different tyrosine kinase cell surface receptors. In this study, well-defined concentration gradients of neurotrophic factors (NFs) allowed us to study the synergistic effect of different NFs (e.g. nerve growth factor [NGF], neurotrophin-3 [NT-3] and brain-derived neurotrophic factor [BDNF]) for axonal guidance of embryonic lumbar dorsal root ganglion cells (DRGs). Effective guidance of DRG axons was achieved with a minimum NGF concentration gradient of 133 ng/ml/mm alone, or combined NGF and NT-3 concentration gradients of 80 ng/ml/mm each. Interestingly, the combined concentration gradients of NGF and BDNF did not show any significant synergism at the concentration gradients studied. The synergism observed between NGF and NT-3 indicates that axons may be guided over a 12.5 mm distance, which is significantly greater than that of 7.5 mm calculated by us for NGF alone or that of 2 mm observed by others.

Key words: neurotrophic factors, concentration gradient, dorsal root ganglion, Trk receptors, nerve guidance.

Axons in developing nervous systems are guided to their targets by a combination of contact-mediated and diffusible cues that are either attractive or repulsive (Cajal, 1928; Crick, 1970; Walsh and Doherty, 1997; Sun et al., 2000). The growth cone is the most active part of the axon at the nerve fiber terminus and is believed to guide axonal growth by sampling its micro-environment for either attractive or repulsive signals using filopodial and lamellar protrusions (Goodman, 1996; Zheng et al.,1996; Mueller, 1999; Seeger and Beattie, 1999). The sampling, comparing and decision making procedure is believed to be a concentration gradient dependent action evoking a set of intracellular events, which further signal the preferential incorporation of new plasma membrane material and asymmetric cytoskeleton reorganization at the growth cone that is required for the appropriate orientation of axons (Keynes and Cook, 1995; Song et al., 1997; Parent and Devreotes, 1999).

Significant research has been devoted to identifying guidance molecules, and recent studies have demonstrated that most of the *neurotrophic* factors, including neurotrophins, are also *neurotropic* (Sun et al., 2000). For example, we previously demonstrated that PC12 cell neurites could be guided by a minimum concentration gradient of nerve growth factor (NGF) for up to 7.5 mm, *in vitro*, using a well-defined concentration gradient in a diffusion chamber (Cao and Shoichet, 2001). Other *in vitro* studies have shown the chemoattractive effect of NGF on neurite outgrowth of embryonic dorsal root ganglion (DRG) neurons (Gundersen and Barrett, 1979). More recently, Song et al. (1997) demonstrated that concentration gradients of brain-derived neurotrophic factor (BDNF) and neurotrophin-3 (NT-3) induced attractive turning responses in *Xenopus* spinal neurons using a micropipette assay. NGF, BDNF and NT-3 interact primarily through different cell surface tyrosine kinase (Trk) receptors (i.e. Trk A, Trk B and Trk C, respectively; Neet and Campenot, 2001). After the pioneering work reported by Farinas et al. (1998) to co-localize the Trk receptors by double immuno-labeling, the co-localization of at least two of these major Trk receptors in DRG neurons have been demonstrated using immunocytochemistry and *in situ* hybridization techniques in both chick embryos and adult rats, respectively (Tuttle and O'Leary, 1998; Karchewski et al., 1999; Rifkin et al., 2000). For example, using double immuno-labeling, Rifkin et al. (2000) observed that sub-populations of cells in the dorsal medial pole were co-localized with either TrkA/TrkC or TrkA/TrkB in embryonic day (E) 10 chick embryos. Thus we hypothesized that there would be a synergistic effect among these growth factors to guide DRG axons among those DRG populations that co-express the Trk receptors.

In the current study, we created a series of stable linear concentration gradients of neurotrophic factors and studied the response of DRG axons in a compartmentalized model system. For the guidance of DRG axons, we observed a synergistic effect using combined NGF and NT-3 concentration gradients relative to a sole NGF concentration gradient. The synergism was quantified by a significant decrease in the NGF concentration gradient threshold

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Abbreviations: BDNF, brain-derived neurotrophic factor; DRG, dorsal root ganglion; E, embryonic day; NGF, nerve growth factor; NT-3, neurotrophin-3; PLL, poly-L-lysine; Trk, tyrosine kinase.

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required to guide axonal growth alone vs. that in the presence of an NT-3 concentration gradient. This lowered concentration gradient threshold suggests the possibility for guidance over a greater distance.

EXPERIMENTAL PROCEDURES

Materials

All chemicals were purchased from Sigma (St. Louis, MO, USA) and used as received unless otherwise indicated. Low gelling temperature SeaPlaque agarose was obtained from FMC Corporation (Rockland, ME, USA). Mouse NGF-β (2.5S NGF) was purchased from Cedarlane Laboratory (Hornby, ON, Canada). Recombinant human NT-3 and recombinant human BDNF were obtained from Promega (Madison, WI, USA). Deionized distilled water was obtained from Milli-RO 10 Plus and Milli-Q UF Plus system (Millipore, Bedford, MA) and used at 18 MΩ resistance.

DRG cultures

Lumbar DRG were dissected from E9 White Rock chicks (Brampton, ON, Canada) according to an established protocol (Nishi, 1996). The dissected DRG tissues were digested with trypsin (0.25%) for 20 min at 37 °C. Individual DRG cells were then obtained by triturating the digested DRG tissues using a flame-polished Pasteur pipette. The resulting DRG cells were then cultured in a basic cell culture medium consisting of 98% Eagle's minimal essential medium (Gibco/BRL), 1% penicillin/streptomycin (Gibco/BRL), 1% N2 supplement (Gibco/BRL) and 0.2 mM glutamine (Gibco/BRL). No neurotrophin was added to the basic cell culture medium to prevent the selection of a particular neuron-subpopulation; instead, N2 supplement, a defined supplement to support the survival and expression of post-mitotic neurons of both the peripheral nervous system and the CNS, was added to the medium. This serum free, N2-supplemented basic medium appears to be selective for neuronal cells and does not support the growth of non-neuronal cells. Cells were incubated at 37 °C in a 5% CO_2 and 95% air atmosphere.

Double-immunocytochemistry

E9 DRGs were cultured in two-well Laboratory-Tek slide chambers (Nalge Nunc) pre-coated with poly-L-lysine (PLL). Cells were cultured overnight in the basic culture medium and fixed with 10% formalin for 10 min. Subsequently, the fixatives were carefully removed and the slide chambers were washed twice with Hanks' balanced salt solution (Gibco/BRL). Immuno-labeling was performed using polyclonal primary antibodies against Trk A (1:200; Santa Cruz Biotech Inc., CA, USA), Trk B (1:200; Santa Cruz Biotech Inc.) and Trk C (1:200; Research Diagnostics Inc., NJ, USA; Davis et al., 1997). In order to assess the co-localization pattern of the high affinity neurotrophic factor receptors (i.e. Trk A, Trk B and Trk C) on the DRG surfaces, fixed DRG samples were first incubated with one primary antibody, followed by incubation with an appropriate fluorescently labeled secondary antibody. The procedure was then repeated accordingly with the second primary antibody specific to a different receptor of interest (therefore investigating the co-expression of two different receptors), followed by a corresponding second fluorescence-labeled secondary antibody for visualization. Specifically, to co-localize the expression of Trk A and Trk C, fixed samples were first incubated overnight at 4 °C with goat anti-Trk C, the first primary antibody, to localize Trk C. This step was followed by thorough and careful washes with Hanks' balanced salt solution. Subsequently, samples were incubated with Alexa Fluor 488 donkey anti-goat IgG (Molecular Probes, OR, USA), the first fluorescence-labeled secondary antibody, at room temperature for 10 min with shaking, and washed thoroughly again with Hanks' balanced salt solution, to visualize the localized Trk C receptors on the cell surface. A similar process was repeated to co-localize Trk A on the same cell surfaces, where rabbit anti-Trk A, the second primary antibody, was incubated with the samples overnight at 4 °C, to localize Trk A. The samples were then washed in the same fashion, and incubated with Alexa Fluor 594 donkey anti-rabbit IgG (Molecular Probes), the second fluorescence-labeled secondary antibody, for 10 min at room temperature with shaking, and washed, to visualize the localized Trk A receptors on the cell surface. Similarly, the co-localization of Trk A and Trk B was performed by consequently applying: 1) goat anti-Trk B and Alexa Fluor 488 donkey anti-goat IgG as the first primary and secondary antibodies, respectively, to localize Trk B; and 2) rabbit anti-Trk A and Alexa Fluor 594 donkey anti-rabbit IgG as the second primary and secondary antibodies, respectively, to localize Trk A. The co-expression of receptors of interest was then visualized with a Nikon Microphot FX fluorescence microscope. (The manufacturers guaranteed the specificity and efficacy of the antibodies.) Control samples were processed without primary antibodies in parallel to control for auto-fluorescence and non-specific labeling by secondary antibodies. No attempt was made to optimize the immuno-labeling by changing the incubation sequence of the primary antibodies (i.e. anti-Trk C then anti-Trk A, anti-Trk B then anti-Trk A).

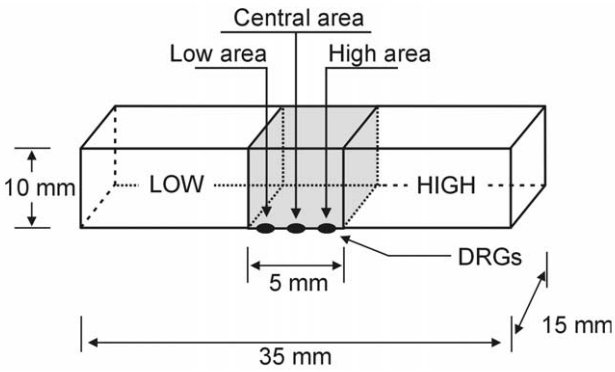

Fig. 1. Compartmentalized diffusion chamber for cell culture. Shaded area (agarose membrane) separates two equivalent compartments, each of which contains a constant, yet different, concentration of NGF or combinations of neurotrophins (i.e. NGF/NT-3 and NGF/BDNF). DRGs are plated beneath the agarose membrane, and cellular responses are observed under three different areas.

Directed axonal outgrowth in compartmentalized culture assay

A 100 mm Petri dish was coated with 10 ml of a PLL solution (36,600 g/mol, 50 μg/ml) for 3 h at room temperature, washed thoroughly with deionized distilled water and then air-dried (Greene, 1977). As shown in Fig. 1, the three-section, autoclaved, rectangular chamber was then glued to the Petri dish using autoclaved high vacuum grease (Campenot, 1994).

Individual DRG cells were plated within the central compartment on the PLL-coated Petri dish at a relatively low cell plating density of 5×10^3 cells/cm^2, to minimize possible cell–cell interaction, and allowed to set for 2 h in the incubator. One half milliliter of 1% agarose gel solution was then cast into the middle compartment, on top of the DRGs. In one set of experiments, high concentration NGF and blank cell culture medium were used in the high concentration (source) and the low concentration (sink) compartments, respectively. Both high and low concentration compartments were replenished every 6 h with fresh NGF and cell culture medium, respectively (medium was changed every 6 h to ensure that the neurotrophic factor remained active and that the

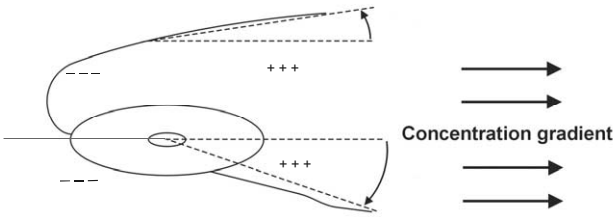

Fig. 2. Neurite orientation is scored relative to the NGF (or NGF/NT-3 or NGF/BDNF) concentration gradient. The angle between an imaginary line from the center of the cell to the high concentration compartment and a line from the center of the cell to the growth cone is utilized to measure orientation. Orientations are measured in degrees, with 0°±90° and 180°±90°, as shown in Fig. 2 with both "+++" and "—" signs, respectively. This symmetrically defined neurite orientation conferred the convenience that highly oriented neurite outgrowth toward the NGF concentration gradient (0° using the above definition) would cluster around and converge on the zero direction.

concentration gradient was stable; see Cao and Shoichet, 2001 for details). A series of NGF concentration gradients were created by using one of: 332 ng/ml (concentration gradient at 66.3 ng/ml/mm) or 665 ng/ml (concentration gradient at 133 ng/ml/mm) NGF in the high concentration compartment and cell culture medium in the low compartment. Stable concentration gradients were established after 30–36 h as previously determined (Cao and Shoichet, 2001), and axon orientation measured after 72 h. For the control, a uniform NGF concentration throughout the agarose was created by inoculating both compartments with the same NGF concentration of 665 ng/ml (i.e. no concentration gradient throughout the gel).

In another set of experiments, the synergistic effect of multiple neurotrophic factors was investigated. Combined neurotrophic factors of NGF and one of NT-3 or BDNF were inoculated at pre-determined concentrations in the high concentration compartment while cell culture medium was added to the low concentration compartment.

After 72 h of culture, axonal growth of DRGs was observed by phase contrast microscopy (Axiovert S100; Zeiss, Germany) for cells in the low, central and high concentration areas. Axons from individual cells were counted in each area, and an axon population of greater than 50 ($n>50$) was analyzed per area (1.2 mm×15 mm, 1.2 mm in the direction of the concentration gradient), for every concentration gradient studied.

To determine guidance, the orientation of axons extending from DRGs was determined by measuring the angle between the axon and the growth factor source compartment. As shown in Fig. 2, the angle measured was between an imaginary line drawn from the center of the cell to the end of the process and an imaginary line from the center of the cell to the high concentration compartment (i.e. up the gradient). For axons that sprouted and turned toward the concentration gradient, the angle was measured between the horizontal (i.e. parallel to the concentration gradient) and the process. Axons that extended from the cell between 0° and ±90° were assigned positive orientations while those extending between 180° and ±90° were assigned negative orientations.

Absolute values were recorded as described more fully in the statistics section. This symmetrically defined neurite orientation conferred the convenience that highly oriented neurite outgrowth toward the NGF concentration gradient (0° using the above definition) would cluster around and converge on the zero direction. All axons that were longer than one cell body length were scored except those that merged with other cells (to eliminate any artifact associated with cell–cell interactions).

Statistics

The axon orientation data that were obtained from the compartmentalized cell culture assay were analyzed, as described in a previous paper (Cao and Shoichet, 2001). Briefly, the data were analyzed first by a uniformity test ($\alpha=0.01$) and then by a conditional unbiased test. (1) The uniformity test was used to determine whether there was any preferred direction to the axonal growth by a χ^2 test. Only those samples that passed the uniformity test were further tested by the unconditional unbiased test to determine the preferred mean direction of axonal growth relative to the neurotrophic factor concentration gradient. (2) The preferred mean direction was then compared with the direction of the concentration gradient (i.e. NF source) with 95% confidence, as described by Mardia (1972). The absolute mean value (with 95% confidence intervals) of preferred mean direction was calculated and is reported herein.

RESULTS

The goals of this study were to investigate the effects of a series of well-defined neurotrophic factor concentration gradients (i.e. dc/dx=constant) on axonal guidance in a compartmentalized cell culture system and to determine whether there was a synergistic effect of combined neurotrophic factor concentration gradients on axonal guidance.

Co-localization of Trk A/B and Trk A/C

It has been established that embryonic chick DRGs can be isolated and cultured *in vitro* between E8 and E12. The DRGs isolated at E9 to E12 produce more robust neurons, with better survival and fewer non-neuronal cells than those isolated at E8 (Nishi, 1996; Smith, 1998). However, the expression of neurotrophin receptors (i.e. Trk A, Trk B, and Trk C) during sensory neuron genesis and differentiation has been shown to be dynamic, changing dramatically during development (Rifkin et al., 2000). Although the co-expression pattern of high affinity neurotrophic factors has been documented in embryonic chick DRGs using immunocytochemistry (Rifkin et al., 2000), we investigated the co-expression pattern of Trk A/Trk B and Trk A/Trk C in the E9 DRGs that we studied. Fig. 3 shows that the expression of Trk A, Trk B and Trk C in DRGs was extensive at E9, as evidenced by the prominent immunostainings in all individual micrographs. More importantly, the comparison of immunostains in Fig. 3A and 3B, as well as those of 3C and 3D showed that the co-expression pattern of Trk A/Trk C and Trk A/Trk B widely existed in DRGs at E9, the embryonic age studied. This result further rationalized and validated our approach to study the synergistic effect among neurotrophic factors to guide DRG axons among those DRG populations that co-express the Trk receptors. No significant immunostains were found in the control samples, where primary antibodies were omitted in the immunocytochemistry analysis, to account for auto-fluorescence and non-specific labeling by secondary antibodies.

Guided axonal growth by concentration gradients of NGF alone

The cellular response of the DRGs to different NGF concentration gradients was studied in terms of axonal orien-

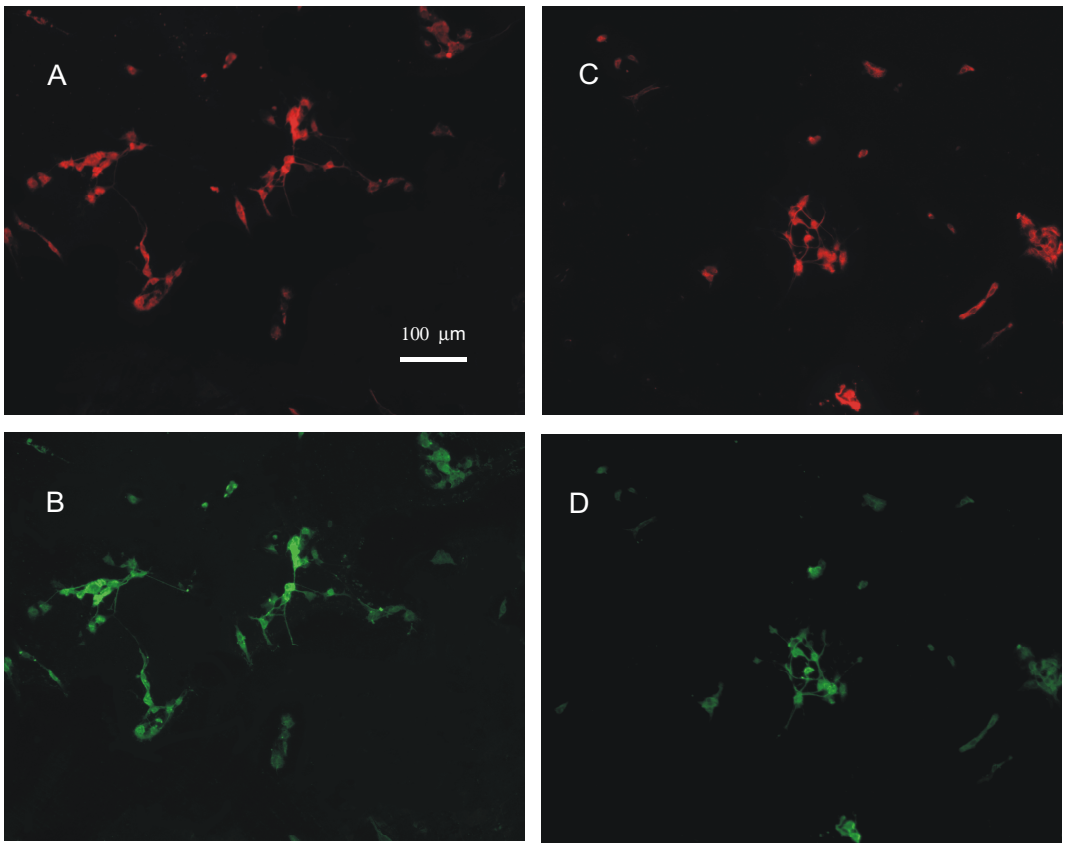

Fig. 3. Immunostaining of Trk A, Trk B and Trk C receptors of chick DRG at E9. (A) Immunostaining of TrK A (shown in red); (B) immunostaining of Trk C of the same frame (as shown in green); (C) Immunostaining of TrK A (shown in red); and (D) immunostaining of Trk B of the same frame (as shown in green). Please note the co-expression of Trk A/Trk C and Trk A/Trk B by comparing stainings in micrographs A/B and C/D.

tation relative to the concentration gradient. In a previous paper (Cao and Shoichet, 2001), we demonstrated that the concentration gradient can be easily designed and maintained in our compartmentalized cell culture model when the concentrations in both compartments are maintained constant. This results in a constant concentration gradient within the agarose membrane (i.e. the concentration profile is linear).

In the compartmentalized cell culture assay, DRGs were plated underneath the agarose gel within which there was a defined NGF concentration gradient. Preferential DRG axonal growth in response to different concentration gradients is summarized in Table 1. No significant guided axonal growth was observed with NGF alone until the NGF concentration gradient reached 133 ng/ml/mm. Interestingly, this was the same concentration gradient required to guide neurite outgrowth from PC12 cells (Cao and Shoichet, 2001).

A representative light micrograph of DRG neurons cultured in the presence of a 133 ng/ml/mm NGF concentration gradient is shown in Fig. 4 It is interesting to note that even when DRG axons did sprout on the opposite side of the concentration gradient (cf. Fig. 4), they always turned toward the gradient for NGF concentration gradients greater than the threshold value. The apparently random sprouting of axons may reflect the experimental design in which it takes some time for the concentration gradient to establish due to the slow passive diffusion of NGF through the agarose membrane. For example, DRGs extend axons within 12 h after exposure to NGF-supplemented growth medium yet the linear concentration gradient is not established until 30–36 h within the agarose gel. Thus the initial sprouting occurs prior to the concentration gradient being established; however, the DRG axons are not scored until 72 h when the NGF is shown to have both *chemotrophic* and *chemotropic* effects.

Guided axonal growth by combined concentration gradients of NGF+NT-3 but not NGF+BDNF

To investigate the possible synergistic effect of different neurotrophic factors for neurotropism of DRG axons, combinations of NGF+NT-3 and NGF+BDNF concentration gradients were tested in the model system. We hypothesized that these factors would act synergistically by stimulating different cell surface receptors (i.e. NGF through Trk A, BDNF through Trk B and NT-3 through Trk C), due to the co-localization of different receptors as shown in the previous text. In order to create dual concentration gradients of NGF and NT-3, a mixture of known concentrations of both NGF and NT-3 were inoc-

Table 1. Effect of neurotrophic concentration gradients, across an agarose membrane, on axonal growth of DRGs (absolute values shown)

NF concentration in low concentration compartment ng/ml	NF concentration in high concentration compartment ng/ml	Absolute NF concentration gradient (dc/dx) (ng/ml/mm)	Preferred direction of axonal growth (in degrees)*§ ($n>50$)		
			Low area	Central area	High area
663 NGF	663 NGF	0.00 (Control)	None	None	None
0.00 NGF	331.5 NGF	66.3 NGF	None	None	None
0.00 NGF	400 NGF	80 NGF	None	None	None
0.00 NGF	665 NGF	133 NGF	14±25	4.1±18	7.9±10
0.00 NT-3	400 NT-3	80 NT-3	None	None	None
0.00 BDNF	400 BDNF	80 BDNF	None	None	None
0.00 NGF	331.5 NGF	66.3 NGF/	None	None	None
0.00 NT-3	500 NT-3	100 NT-3			
0.00 NGF	400 NGF	80 NGF/	7.6±12	4.9±11	12±17
0.00 NT-3	400 NT-3	80 NT-3			
0.00 NGF	331.5 NGF	66.3 NGF/	None	None	None
0.00 BDNF	500 BDNF	100 BDNF			
0.00 NGF	400 NGF	80 NGF/	None	None	None
0.00 BDNF	400 BDNF	80 BDNF			

* Determined by χ^2 test ($\alpha=0.01$, $n>50$).
§ Mean±95% confidence interval.

ulated in the high concentration compartment and cell culture medium was added to the low concentration compartment. Similar methodology was followed for NGF+BDNF. The data, summarized in Table 1, show guided axonal growth at a dual concentration gradient of 80 ng/ml/mm NGF and 80 ng/ml/mm NT-3 (referred to in Table 1 as 80 NGF/80 NT-3); in contrast, when each was applied separately, neither the NT-3 concentration gradient at 80 ng/ml/mm nor the NGF concentration gradient at 80 ng/ml/mm induced any significant guided neurite outgrowth. The observation that significant guided neurite outgrowth was observed when NGF and NT-3 concentration gradients were combined (i.e. 80 NGF/80 NT-3), while no preferred directional growth was evident when either concentration gradient was applied separately, strongly suggests that there is a synergistic effect of NGF and NT-3 to guide neurite outgrowth at an appropriate combined concentration gradient. No significant guided axonal growth was observed at either combination of the NGF+BDNF concentration gradients studied. Interestingly, DRG survival improved after 3 days in culture for both NGF/NT-3 and NGF/BDNF relative to NGF alone. Micrographs, as shown in Fig. 5a–f, are a collection of DRGs observed in the high, central and low areas for both NGF/NT-3 (80/80) and NGF/BDNF (80/80), respectively.

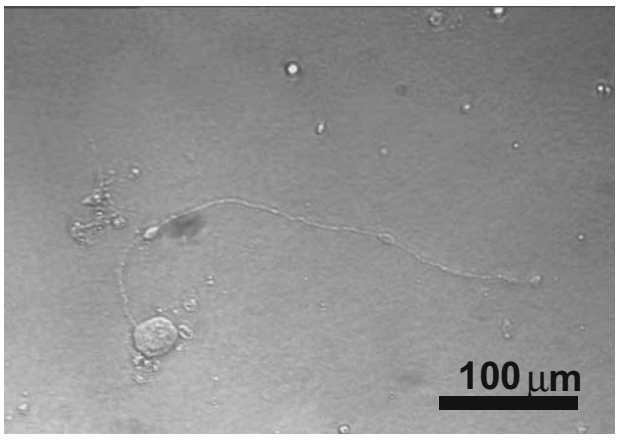

Fig. 4. Axonal outgrowth of DRGs up the concentration gradient was evident after 72 h in culture.

DISCUSSION

The compartmentalized model system provides a unique approach for studying the effect of guided axonal growth by a concentration gradient of neurotrophic factor(s). By maintaining the concentrations in both high and low concentration compartments nearly constant, a well-defined concentration gradient was designed and prepared as desired (Cao and Shoichet, 2001). This model system allowed us to study the possible synergistic effect of combined neurotrophic factors, and perhaps more importantly to quantify this synergism (for the first time) by investigating whether the threshold value for guidance by one factor (i.e. NGF) can be lowered in the presence of a second concentration gradient (i.e. NT-3 or BDNF). Our well-defined model contrasts with others where concentration gradients of guidance molecules were created by micro-pipetting from a point source (Gundersen and Barrett, 1979). Thus little is known about the gradients required for guidance with molecules, such as epherins, netrins and semaphorins. Our experimental setup also allowed us to determine whether guidance can be achieved over a longer distance due to the synergism of two factors vs. one. Growth cones will navigate over long distances if: (1) the concentration of a given neurotrophic factor does not saturate cell surface receptors, which could be consequently down regulated (Zigmond, 1981) and (2) the concentration gradient is sufficiently steep for growth cone detection (Goodhill, 1997). We previously reported (Cao and Shoichet, 2001) that PC12 cell neurites could be guided by

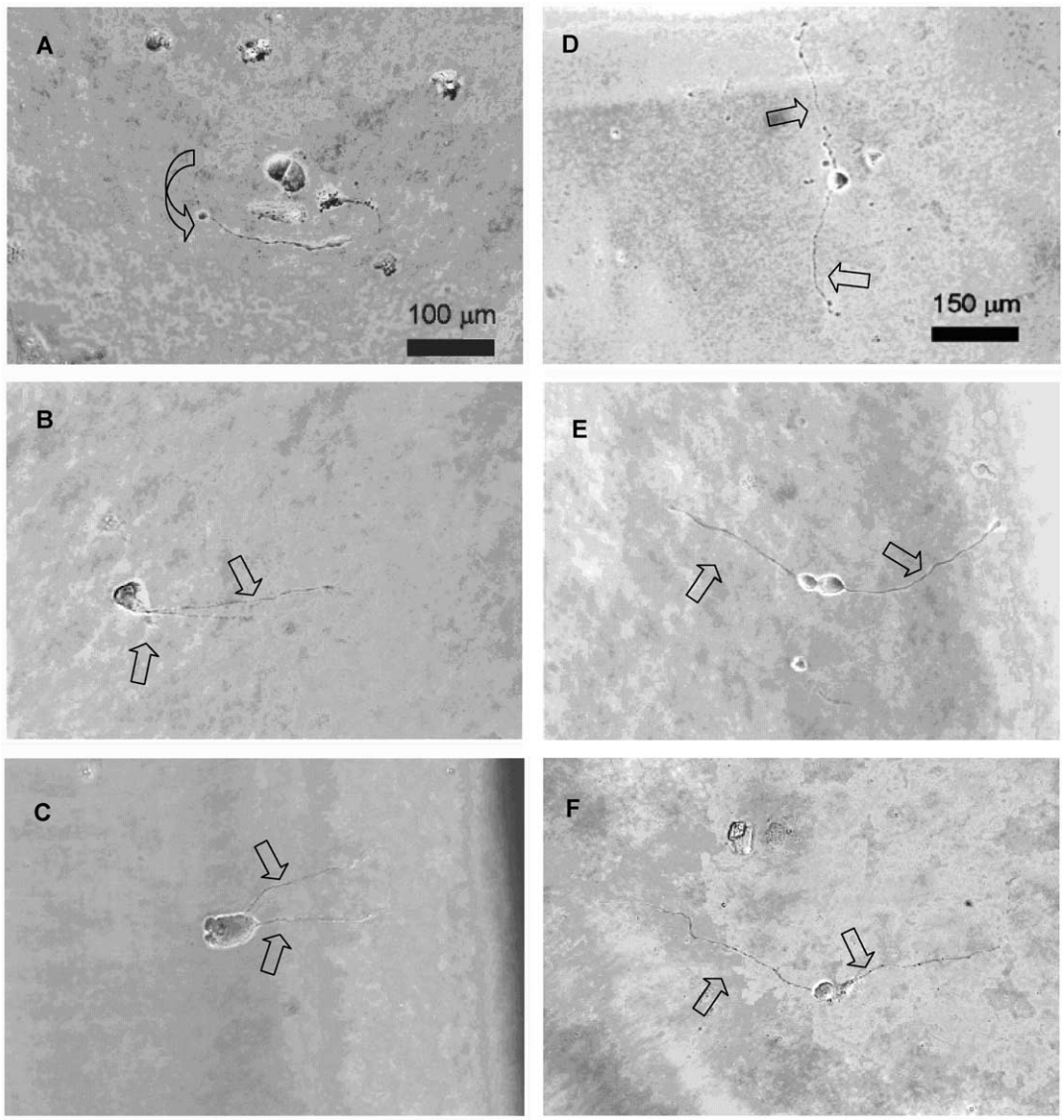

Fig. 5. A collection of representative DRG neurons observed in the high, central and low areas. A–C demonstrate synergistic effect of NGF/NT-3 (80/80) concentration gradients in guiding DRG axons after 72 h; D–F demonstrate no significant guidance effect by concentration gradients of NGF/BDNF (80/80). Note the differences in the neurite orientations with or without effective guidance and pay special attention to DRG bipolar morphology as indicated by arrows. Figures A/D, B/E, and C/F are micrographs taken in the low, central and high areas. Concentration gradient is from the left to the right across the micrographs.

a NGF concentration gradient alone over 7.5 mm, which differed from previous reports of guidance ranging from 0.5 mm to 2 mm (Goodhill, 1997; Wu et al., 1999). We attributed our success to both the linear concentration profile and homogeneous concentration gradient achieved throughout the gel.

In this study, we repeated these studies with primary DRGs in place of PC12 cells, taking advantage of the fact that DRGs co-express Trk receptors A, B and C, as we demonstrated in the immunocytochemistry analyses. This allowed us to investigate whether multiple neurotrophic factors could be used to both increase the guidance distance and lower the concentration gradients required for guidance. We found that DRG neurons require a similar NGF concentration gradient for guidance over a similar distance ($<$7.5 mm) as PC12 cells when NGF alone provides neurotropism. This similar guidance pattern for both cell types likely indicates the importance and presence of the Trk A cell-surface receptors through which the NGF concentration gradient primarily exerts its neurotropic effect (Reichardt and Farinas, 1999). In the presence of dual concentration gradients of NGF+NT-3, the threshold NGF concentration gradient for axonal guidance was lowered from 133 ng/ml/mm to 80 ng/ml/mm, likely indicating that both Trk A and Trk C receptors are stimulated and provide neurotropism. Conversely, in the presence of a dual concentration gradient of NGF+BDNF, no significant synergism was observed for axonal guidance. This observation agrees well with the study conducted by Ming et al. (1999) in which the growth cone turning behavior of *Xenopus*

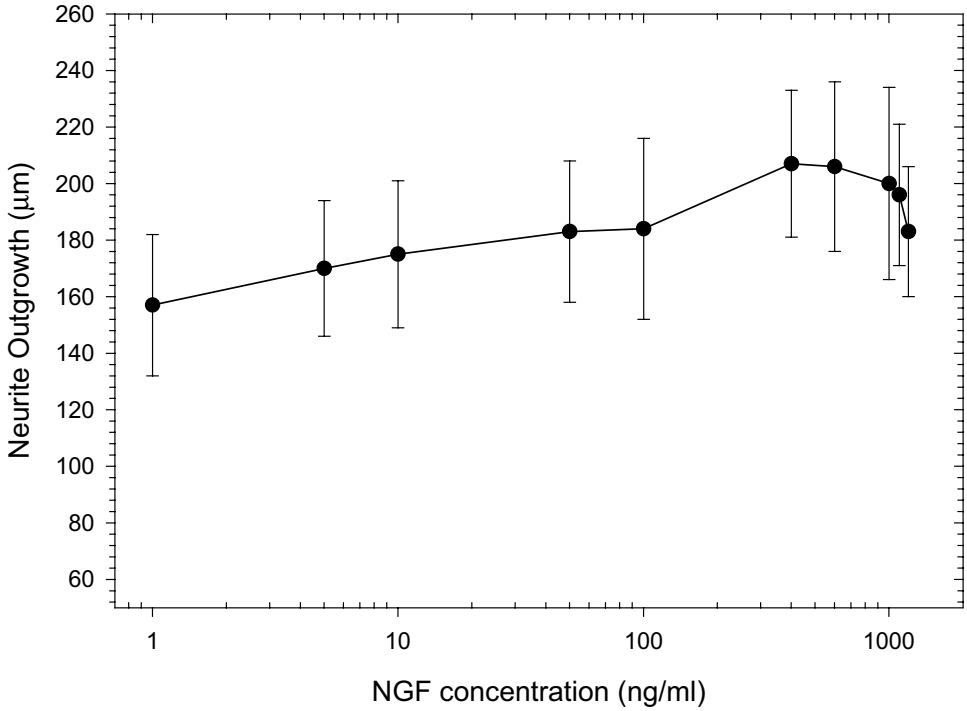

Fig. 6. Dose-dependence of DRG axonal outgrowth at different concentrations of NGF with 400 ng/ml NT-3 as background after 48 h of culture. Data in the figure are presented as median, and error bars indicate a range from 25th percentile to 75th percentile.

spinal neurons was guided by a concentration gradient of NT-3 in the presence of NGF but not by a concentration gradient of BDNF in the presence of NGF. This, Ming and his colleagues (1999) argued, may be explained by BDNF and NGF sharing common cytosolic signaling pathways for guidance cues whereas NT-3 and NGF use two separate pathways for guidance (Ming et al., 1999; Song and Poo, 1999). They also classified soluble guidance cues into two groups: group I includes guidance cues such as NGF, BDNF and netrin while group II includes molecules such as NT-3 and Sema III. Our observations, together with those of Ming et al. (1999) and Song and Poo (1999), may suggest that guidance cues activate common signaling pathways within each group and that the signaling event is modulated by second messengers, such as cAMP and cGMP, in response to guidance cues (Garrity, 1999). However, it is not yet clear how the synergistic effect is triggered by guidance cues from different groups (Sun et al., 2000).

Our experiments could not decisively exclude the possibility that the observed synergy between NGF and NT-3 is due to the promiscuity of NT-3, which has been shown to also interact with Trk A and Trk B. However this is unlikely since NT-3 binds with Trk A and Trk B at a considerably lower affinity than Trk C (Davies, 2000; Neet and Campenot, 2001). Although it has been shown that the p75 receptor, a pan-neurotrophin receptor which binds NGF, BDNF and NT-3 with considerably low affinity, is involved in modulating axonal growth and axonal guidance (Yamashita et al., 1999; Davies, 2000), it is unlikely that p75 modulation plays a significant role in the synergy observed in this study given that the combination of NGF and BDNF did not elicit significant guidance.

In this study, we demonstrate the extensive co-expression of Trk receptors using double immuno-staining *in vitro*, and show that approximately 60% to 70% of the DRGs at E9 co-express either TrkA/TrkB or TrkA/TrkC. Although our data strongly suggest that there is a certain DRG sub-population that co-expresses all three known Trk receptors (since the sum is greater than 100%), we have not studied this because we are interested in co-expression of Trk A/Trk B and Trk A/Trk C to follow the potential synergism of NGF/BDNF and NGF/NT-3, respectively. While the data strongly support synergism, we cannot exclude the possibility that the neurotropism observed may be due to two sub-populations of neurons that are each TrkA-responsive only and TrkC-responsive only.

The synergies observed for NGF and NT-3 result in a significantly reduced NGF threshold concentration gradient required for guidance. Although very little is known about how a threshold concentration gradient is used for axonal guidance, recent studies on the servomechanism model (Loschinger et al., 2000) and the computational analysis developed therefrom suggest that there seems to be an intrinsic threshold of receptor activity with which a guidance cue is compared. Our results with NGF and NT-3 indicate that cells adjust the threshold for guidance in response to multiple synergistic guidance cues. However, the mechanism remains unclear.

The reduced NGF threshold concentration gradient required for axonal guidance in the presence of NT-3 suggests that axons can be guided over longer distances than

the estimated 7.5 mm by a NGF concentration gradient alone. As shown in Fig. 6, DRG neurite length generally increases with NGF concentration to 600 ng/ml and then plateaus to 1000 ng/ml, after which neurite length decreases, possibly because the NGF receptor on the DRGs are depleted due to receptor down regulation (Zigmond, 1981). Interestingly, the saturation profile for DRGs is similar to that of PC12 cells (Cao and Shoichet, 2001), which may suggest that both cell types have similar amounts of Trk A receptors. When we divide 1000 ng/ml (upper limit in NGF concentration prior to saturation) by 80 ng/ml/mm (NGF gradient required for axonal guidance in the presence of NT-3), we calculate the distance over which guidance can be expected to be 12.5 mm, which is greater than that of 7.5 mm, previously calculated for NGF alone. This 12.5 mm is an estimate of the maximum range that these chemotactic cues will be effective. Recent results by Zhang and Kuffler (2000) support this hypothesis for guidance over greater distances with multiple stimuli. They showed that adult motor axons were guided over a distance $>$6.5 mm by denervated peripheral nerve-conditioned media *in vivo* in frogs. Denervated peripheral nerves have been shown to release a number of factors with potent neurotrophic properties (Zhang and Kuffler, 2000) that may act synergistically to guide axonal growth over an extended distance even when concentration gradients of such factors are ill defined. Interestingly, Jerregard et al. (2001) recently demonstrated the trophic and tropic influences of target-derived molecules from both skin and muscle, on regenerating motor and sensory axons over distances of 8–10 mm. Furthermore, Isacson and Deacon (1996) showed that long-distance axon guidance is also a necessary process for re-connecting specific circuitries in adult central nervous systems after injury.

CONCLUSIONS

A synergistic guidance effect was observed for DRG axons in response to the dual concentration gradients of NGF+NT-3, each having a lower concentration gradient than NGF alone. This extends our predictions for the effective guidance range by a target tissue releasing neurotropic factors during neurogenesis. Furthermore, since regenerating neurons also rely on concentration gradients of neurotrophic factors to re-innervate their targets, the synergistic guidance effect may also be employed to enhance nerve regeneration and functional recovery. We aim to immobilize these well-defined concentration gradients within a device that will be implanted into the spinal cord to enhance repair and recovery (Cao and Shoichet, 2002).

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