

# The Response of Dorsal Root Ganglion Axons to Nerve Growth Factor Gradients Depends on Spinal Level

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## Abstract

Directed sensory axon regeneration has the potential to promote functional recovery after peripheral nerve injury. Using a novel guidance assay to generate precisely controllable nerve growth factor gradients, we show for the first time that the guidance and outgrowth response of rat dorsal root ganglion neurons to identical nerve growth factor gradients depends on the rostrocaudal origin of the dorsal root ganglion explant. These findings have implications for the study of peripheral nerve regeneration in response to exogenous neurotrophins such as nerve growth factor, and provide new insight into the clinical potential of nerve growth factor in the treatment of nerve injury.

**Key words:** axon guidance; dorsal root ganglion; nerve growth factor; regeneration; spinal level

## Introduction

**N**ERVE INJURY is associated with significant morbidity due to the inability of neurons to appropriately reconnect with their targets. A therapeutic aim after peripheral nerve injury is thus to re-establish connectivity by aiding in axonal regeneration and correct axonal guidance. Neurotrophic factors such as nerve growth factor (NGF) are of clinical interest, as they promote neuronal survival and provide trophic support to prenatal and adult neurons (Verge et al., 1996). Furthermore, when present in gradients, NGF functions as a neuronal guidance cue, and may thus be useful in promoting regrowth of peripheral axons to the correct targets (Gundersen and Barrett, 1979; Letourneau, 1978; Moore et al., 2006; Rosoff et al., 2004). In the treatment of peripheral nerve injury, administration of NGF increases nerve regeneration and prevents axotomy-induced neuronal changes, highlighting the therapeutic potential of neurotrophins for enhancement of functional recovery (Savignat et al., 2008; Verge et al., 1996).

The level of the spinal cord at which injury occurs is crucial in determining functional losses and physiological effects. However, the effect of spinal level on NGF-mediated neurite regeneration and the guidance response of peripheral neurons to gradients of NGF have not been systematically assessed. Such information is important for the *in vitro* study of peripheral nerve regeneration in response to NGF, and also for assessing the therapeutic potential of NGF based on the level of the spinal cord at which injury occurred. Limitations of

available guidance assays (Pujic et al., 2009), such as the difficulty of precisely controlling gradient steepness and concentration at the explant, have precluded systematic assessment to date. We recently introduced a novel guidance assay allowing generation of precisely controlled gradient conditions (Mortimer et al., 2009; Rosoff et al., 2005, 2004). Using this assay we have shown that, averaging over dorsal root ganglia (DRG) from several spinal levels, the effect of exquisitely small differences in gradient parameters can be seen in differential guidance responses of DRGs to NGF gradients (Mortimer et al., 2009). Using this assay we now show that for identical gradient parameters, the guidance and outgrowth response of DRG explants to NGF gradients varies significantly depending on spinal level. This has important implications not only for the *in vitro* study of NGF in peripheral nerve regeneration, but also for the application of NGF gradients to the treatment and prognosis of nerve injury *in vivo*.

## Methods

### Neurite guidance assay

Experiments involving animals were conducted in accordance with the Animal Care and Protection Act Qld (2002), and the *Australian Code of Practice for the Care and Use of Animals for Scientific Purposes*, 7th edition (2004). Ethics approval was obtained from the University of Queensland Ethics Committee.

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The neurite outgrowth (OG) and guidance response (GR) of DRGs to NGF gradients was assessed as previously described (Mortimer et al., 2009, 2010; Rosoff et al., 2004, 2009). This assay is summarized in Figure 1. For a precise description of the protocol, see Rosoff and associates (Rosoff et al., 2009). In brief, DRGs were isolated from the sacral (S3) to cervical (C8) spinal levels of a total of 18 Wistar rats (P0–P2) and kept separate at all times. DRG explants were prepared by trimming nerve roots using microsurgical vannas scissors, followed by loosening of the connective tissue sheath by incubation with 0.25% trypsin at 37°C for 5 min. After further removal of the remaining nerve roots and connective tissue, the DRG explants were transferred to Hibernate-E medium (Brain Bits, Springfield, IL) and kept at 4°C until plating in dry collagen gel. Only intact DRG explants were used.

#### Preparation of dry collagen gel

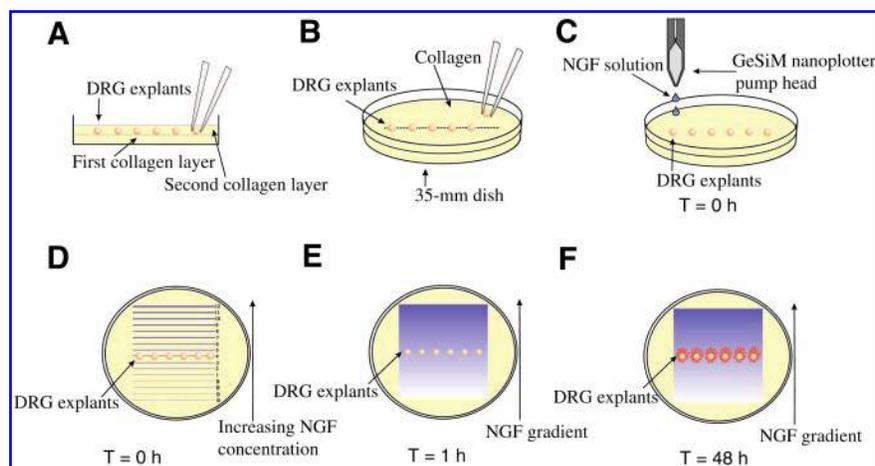
Collagen gels were prepared as previously described (Mortimer et al., 2009; Rosoff et al., 2009, 2004). In brief, rat tail collagen (BD Biosciences, San Jose, CA) was mixed on ice with 27  $\mu\text{L}$  of a 7.5% sodium bicarbonate solution per milliliter of collagen, penicillin, streptomycin, and amphotericin B, and diluted with appropriately concentrated OptiMEM solution to result in 0.2% collagen in 1 $\times$ concentrated OptiMEM. To form the bottom layer of the gel (Fig. 1A), 750  $\mu\text{L}$  of this solution was evenly spread on 35-mm dishes and allowed to set. A second layer of 750  $\mu\text{L}$  of collagen was added and 6 DRG explants were precisely placed in a line within this second layer using a custom-made acrylic dish holder (Fig. 1B). Prior to printing of molecular gradients as described below, the plates were briefly transferred to the incubator to allow for complete setting of the gel matrix.

#### Gradient generation

Precise gradients of NGF (PeproTech, Rocky Hill, NJ) were generated essentially as previously described by Mortimer and colleagues (2009). Briefly, a GeSiM Nanoplotter (GeSiM mbH, Groberkmannsdorf, Germany) was used to print 12 lines of NGF solutions with exponentially increasing concentration on top of the collagen gel (Fig. 1C and D). The NGF diffuses into the collagen gel, thus creating an NGF concentration gradient that is stable for 2 days, as previously demonstrated using fluorescently-labeled casein (Rosoff et al., 2004). The gradient concentration parameters were adjusted to result in a 0.3% increase in NGF concentration over the width of a growth cone (10  $\mu\text{m}$ ; Fig. 1E). The NGF concentration at the line corresponding to the explant position was 0.3 nM. Our previous work shows that (averaged over DRGs from many spinal levels) these parameters produce strong outgrowth and guidance (Fig. 1F). While variations in explant size could induce very small variations in the effective diffusion constant of the NGF in the gel, such variations would not affect the final gradient shape (Crank, 1975). After printing of NGF gradients, the plates were incubated for 40–48 h at 37°C with 5% CO<sub>2</sub> before fixation and staining as described below.

#### Immunofluorescence

The explants were fixed in 10% formaldehyde and 0.1% Triton-X 100 in PBS for several hours. The dishes were washed five times for 1 h each with 5 mL PBS between each incubation step. For visualization of neuron-specific  $\beta$ 3-tubulin, the explants were incubated overnight with 0.5  $\mu\text{g}/\text{mL}$  mouse monoclonal TUJ1 antibody (R&D Systems, Inc., Minneapolis, MN), followed by overnight incubation with Alexa-Fluor 488-conjugated secondary anti-mouse antibody (Invitrogen,



**FIG. 1.** Schematic diagram illustrating the steps and procedures involved in the 3D chemotaxis assay used to assess neurite outgrowth and guidance in response to NGF gradients. (A) DRG explants were embedded in a layer of liquid collagen solution pipetted on top of a preformed bottom collagen layer in 35-mm dishes. (B) Using an acrylic 35-mm dish holder with a gradient template, the DRG explants were precisely positioned in the appropriate coordinates within the collagen gel. (C and D) At  $T = 0$  h, four lines of buffer solution and 12 lines of increasingly concentrated NGF solution were printed on top of the collagen gel using a GeSiM Nanoplotter (the lines of buffer prevent a discontinuity in collagen solution density at the low end of the gradient). (E) The gaps between the lines of NGF were rapidly filled in by diffusion over a period of about 1 h, leading to a smooth gradient of NGF. However, at the position of the explants in the gel, both the concentration and gradient steepness remained stable over the following 2 days. (F) DRG explants were incubated for 40–48 h at 37°C with 5% CO<sub>2</sub> before fixation and staining (NGF, nerve growth factor; DRG, dorsal root ganglia). (Color image is available online at [www.liebertonline.com/neu](http://www.liebertonline.com/neu)).

Carlsbad, CA). Explants were imaged using a Zeiss Z1 microscope.

#### Image analysis

Immunofluorescent images of TUJ1-stained DRG explants were processed using Adobe Photoshop. After thresholding to produce a binary image, the total number of pixels in neurites and the explant body was determined. Outgrowth was defined as the total number of neurite pixels divided by the total number of explant pixels. This accounted for differences in explant size and normalized the outgrowth measure to explant size. The guidance ratio was determined from  $(H - L)/(H + L)$ , where H is the number of neurite pixels on the high-concentration side of the explant, and L is the number of pixels of the low-concentration side of the explant. As this guidance ratio measure is normalized to the total neurite pixels, it is relatively insensitive to total neurite outgrowth (Rosoff et al., 2004).

#### Tropomyosin-related kinase A (TrkA) immunohistochemistry

DRG cryostat sections ( $14\ \mu\text{m}$ ) from freshly isolated DRG explants or DRG explants maintained in culture for 48 h were blocked with 2% normal goat serum, followed by incubation with antibodies against TrkA (1:1000; Millipore, Billerica, MA), and TuJ1 (1:1000; R&D Systems), and then with secondary antibodies (anti-mouse Alexa-488 and anti-rabbit Alexa-546; Molecular Probes, Eugene, OR). After counterstaining with DAPI, the sections were cover-slipped and imaged on a Zeiss Z1 microscope (Carl Zeiss, Jena, Germany). To

quantitatively compare TrkA expression based on immunohistochemical studies, 3–12 DRG sections from a minimum of three dorsal root ganglia per spinal level were analyzed.

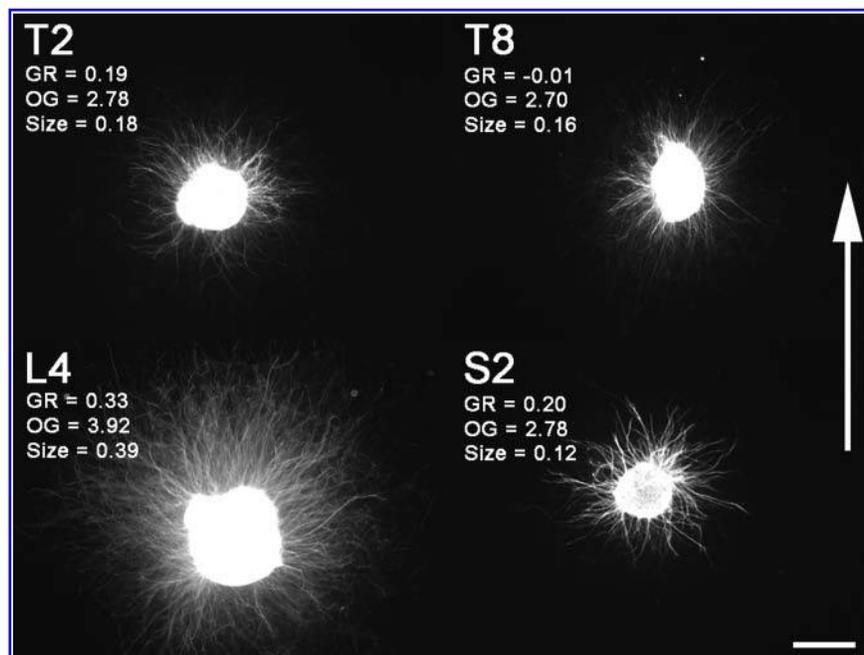
#### Western blotting

Freshly isolated DRGs or DRGs cultured for 48 h were homogenized in 20 mM Tris (pH 8.0), 137 mM NaCl, 0.1% SDS, 2 mM EDTA, 2 mM EGTA, and a proteinase inhibitor cocktail (Roche Diagnostics Corp., Indianapolis, IN). Samples ( $n = 3\text{--}5$  per spinal level) were resolved by SDS-PAGE (4–12% gradient gels; Invitrogen), and transferred to PVDF membranes. After blocking in 5% non-fat milk in PBST, the blots were incubated with anti-TrkA and anti- $\beta$ -actin antibodies. Following washes in PBST, the membranes were incubated with horseradish peroxidase-conjugated secondary antibodies, and processed using enhanced chemiluminescence (Pico West Kit; Pierce Protein Research Products, Rockford, IL) for freshly isolated DRG protein.

For DRG protein isolated after 48 h of culture in the presence of NGF, the membranes were incubated with both anti-mouseIR700 and anti-rabbitIR800CW antibodies, washed as before, and then scanned with a LI-COR Odyssey Infrared Imaging System scanner (LI-COR Biotechnology, Lincoln, NE). Band intensities were quantified using ImageJ software (National Institutes of Health).

#### Real time RT-PCR

For measurement of rat TrkA and  $\beta$ -actin mRNA using quantitative real time RT-PCR, total RNA was isolated from freshly isolated DRGs ( $n = 3$  per spinal level), or DRGs



**FIG. 2.** DRGs from different spinal levels show differential responses to shallow gradients of NGF in collagen. Representative immunofluorescent images of TUJ1-stained DRG explants exposed to a 0.3% NGF gradient with 0.3 nM at the explant. The arrow indicates direction towards increasing NGF concentration. T2, T8, L4, and S2 refer to spinal level (GR, guidance ratio; OG, outgrowth (normalized by explant size), Size = DRG area in mm<sup>2</sup>). The values of GR, OG, and size for each of the explants shown are close to average values for the levels they represent (scale bar = 400  $\mu\text{m}$ ; NGF, nerve growth factor; DRG, dorsal root ganglia).

cultured for 48 h ( $n=3$  per spinal level), using Trizol (Life Technologies, Gaithersburg, MD), and 70 ng of RNA was reverse transcribed with the SuperScript III First-Strand Synthesis System (Invitrogen). Each RT reaction was amplified using the Platinum SYBR Green qPCR Supermix Kit (Invitrogen) according to the manufacturer's instructions. Samples were incubated in a Rotor Gene 3000 cyclor (Corbett Research Pty Ltd, Sydney, Australia) for an initial denaturation at 95°C for 15 min, followed by 40 PCR cycles, consisting

of 95°C for 10 sec, 60°C for 15 sec, and 72°C for 20 sec. Primer sequences for rat TrkA were forward: ATGGAGAACCCA CAGTACTTC, and reverse: CGTGCAGACTCCAAAGAA GC. Primer sequences for rat  $\beta$ -actin were forward: CAG CCTTCCTTCCTGGGTA, and reverse: ATAGAGGTCTTT ACGGATGTC. SYBR Green 1 fluorescence emissions were monitored after each cycle. Amplification of specific transcripts was confirmed by melting curve profiles at the end of each PCR.

#### Statistical analysis

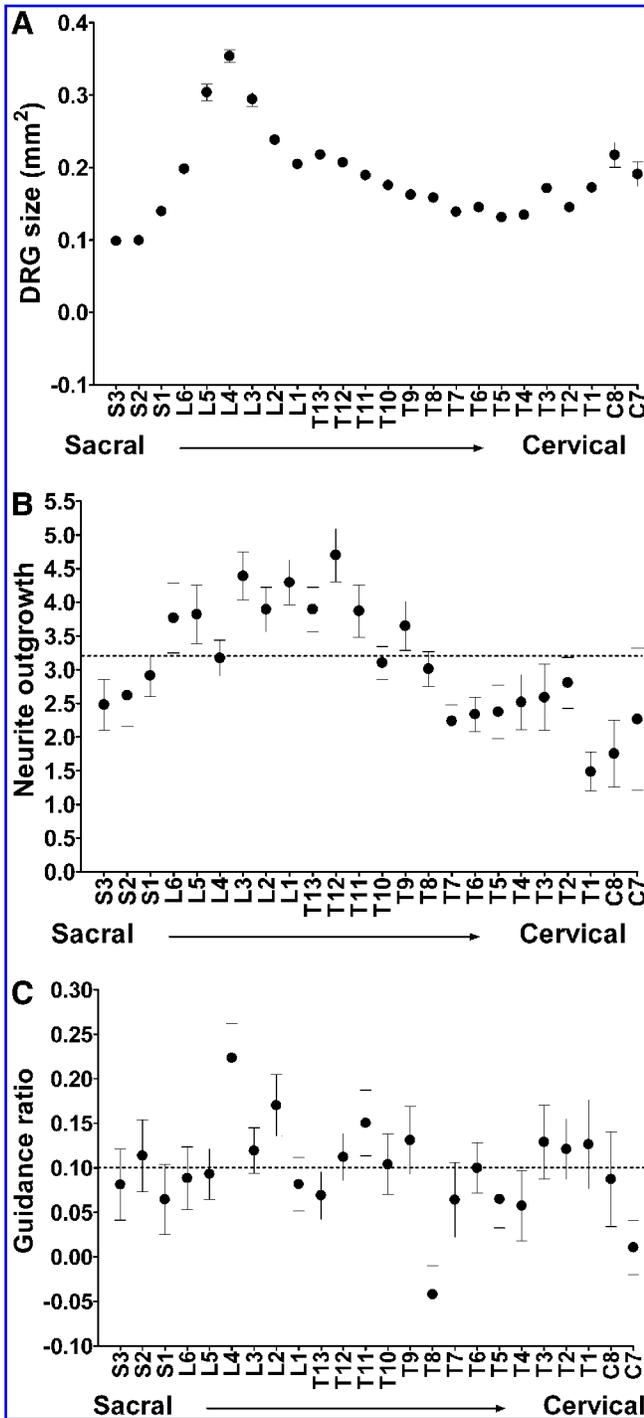
As detailed in Mortimer and colleagues (2009), explants with outgrowth measure  $<1$  (that is, explants with low outgrowth for which the number of neurite pixels was less than the number of explant pixels) were excluded from guidance ratio analysis. Data are presented as mean  $\pm$  SEM unless otherwise stated. For each spinal level,  $n=6$ –24 DRGs were analyzed (i.e.,  $n=18$ –72 per group of three). Statistical significance was set at  $p < 0.05$  and determined using ANOVA,  $t$ -test, or Mann-Whitney tests unless otherwise stated.

## Results

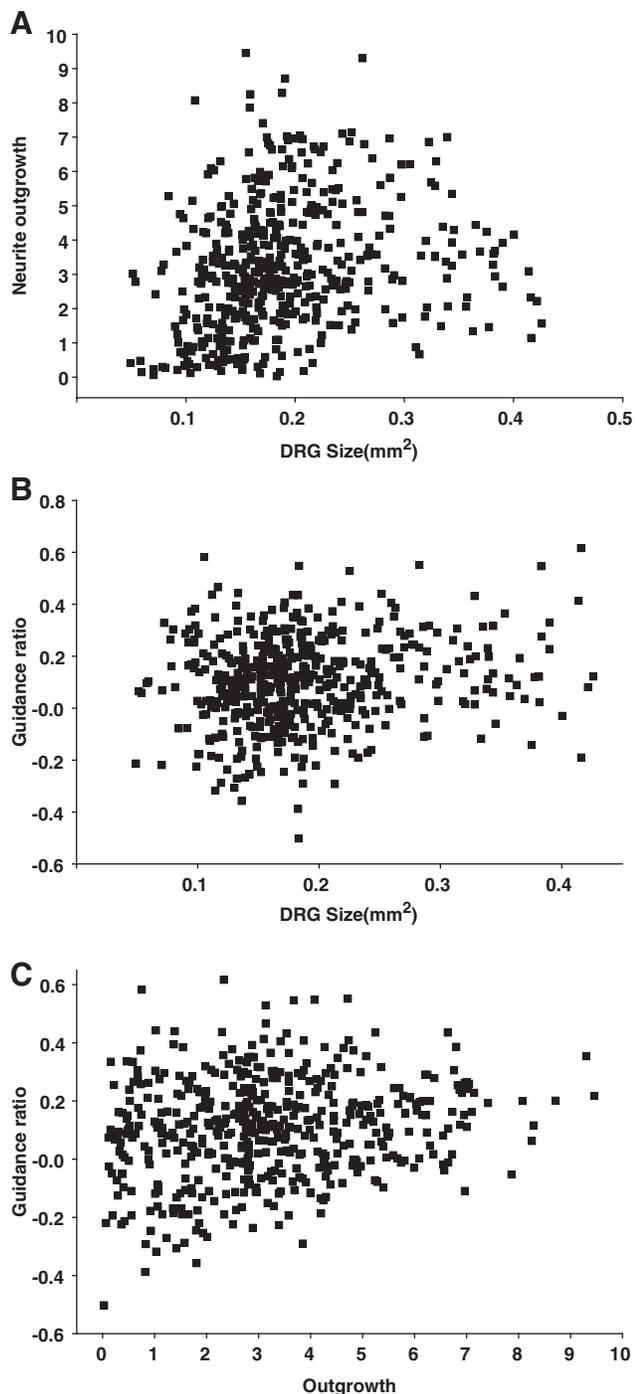
### Neurite outgrowth and guidance

Using the guidance assay of Rosoff and associates (2004; 2005), as updated by Mortimer and colleagues (2009), DRGs isolated from spinal levels S3–C8 were exposed to identical NGF gradients. The gradient parameters were a steepness of 0.3% per 10 microns, and an NGF concentration of 0.3 nM at the explant. Representative explants are shown in Figure 2. As expected (Goldstein et al., 1995; Hamburger and Levi-Montalcini, 1949), explant size was dependent on spinal level, with a sharp peak occurring at level L4 (Fig. 3A).

Neurite outgrowth in response to NGF was measured by dividing the number of neurite pixels by the number of explant pixels (Mortimer et al., 2009; Rosoff et al., 2004). This outgrowth measure removes the effect of larger explants tending to have larger outgrowth simply because they contain more neurons, leaving only variations in outgrowth that are independent of explant size. Increased fasciculation, although not quantified, was observed in DRG explants from sacral regions. This effect could in principle lead to a small effect on



**FIG. 3.** Average response of DRGs to NGF as a function of spinal level. Response of DRG explants to 0.3%/0.3 nM gradients of NGF ( $n=11$ –24 DRG explants from 18 animals). (A) Explant size depends on the rostrocaudal location of the DRG. (B) Neurite outgrowth normalized by explant size in response to exogenous NGF varied significantly (ANOVA,  $p < 0.0001$ ) across spinal levels, with peak outgrowth occurring at levels T7–L6. The average level is indicated by the dashed line. (C) Directed growth up the gradient (as measured by the guidance ratio) varied significantly across spinal levels (ANOVA,  $p < 0.05$ ), with above average guidance at L2 ( $t$ -test,  $p = 0.005$ ), and L4 ( $p = 0.04$ ), and below average guidance at T8 ( $p = 0.001$ ). The average level is indicated by the dashed line. A two-tailed  $t$ -test for C7 compared to the average level of guidance gave  $p = 0.054$ ; thus C7 was also close to having significantly lower guidance than average (NGF, nerve growth factor; DRG, dorsal root ganglia; ANOVA, analysis of variance).



**FIG. 4.** Explant size does not correlate with neurite outgrowth or guidance response. Neurite outgrowth and guidance ratio in response to exogenous NGF gradients are discrete responses, and are independent of physical characteristics such as explant size. In each panel there is one point per explant (all spinal levels are represented). (A) Neurite outgrowth (normalized by explant size) versus explant size ( $r^2 = 0.06$ ). (B) Guidance ratio versus explant size ( $r^2 = 0.02$ ). (C) Guidance ratio versus outgrowth ( $r^2 = 0.04$ ; NGF, nerve growth factor).

the quantitation of outgrowth. Nonetheless, as our outgrowth measure relies on pixel number rather than the identification and tracing of individual neurites, such an effect can be expected to be negligible. Indeed, as is obvious from Figure 2A and D, the neurite length from T2 DRG explants is approximately comparable to the neurite length of S2 DRG explants ( $\sim 350\text{--}450\ \mu\text{M}$  on the high NGF side), and accordingly, the outgrowth measure of T2 and S2 DRG explants is very similar.

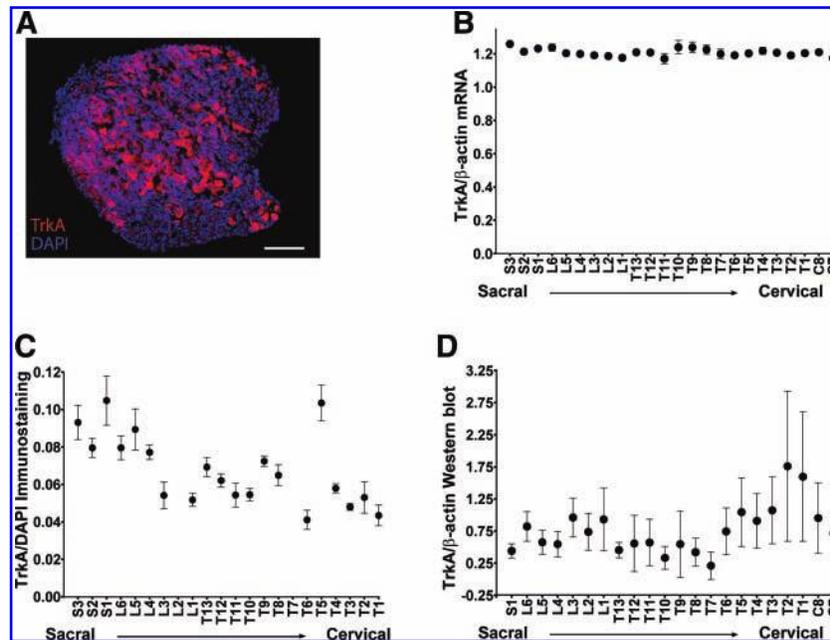
The average neurite outgrowth across all spinal levels was  $3.2 \pm 0.1$  ( $n = 506$ ), which was significantly increased compared to explants in the absence of NGF ( $1.9 \pm 0.2$ ;  $n = 53$ ;  $p < 0.05$ ). However, neurite outgrowth in response to NGF was not uniform across spinal levels. DRG explants from lumbar and lower thoracic levels, specifically L6–T7, responded with large outgrowth to NGF (e.g., outgrowth at L3 =  $4.4 \pm 0.3$ ), with neurite outgrowth declining in DRGs from sacral and thoracic levels (e.g., outgrowth at T6 =  $2.2 \pm 0.2$ ; Fig. 3B).

Guidance responses of DRG neurons to NGF gradients were measured using the guidance ratio (GR), which computes neurite growth up the concentration gradient compared to neurite growth down the gradient (Mortimer et al., 2009; Rosoff et al., 2004). A GR of 0 represents equal neurite growth on the up and down sides of the explant. DRG explants responded to NGF gradients with positive GR on average (Fig. 3C). The overall GR was  $0.1 \pm 0.01$  ( $n = 440$ ), confirming attraction by the gradient. However, the GR was not uniform across spinal levels (Fig. 3C). Specifically, DRGs from L2 and L4 had above average guidance (GR at L2 =  $0.19 \pm 0.02$ ; GR at L4 =  $0.22 \pm 0.04$ ;  $p < 0.05$ , Wilcoxon signed-rank test), while DRGs from T8 had below average guidance (GR at T8 =  $-0.04 \pm 0.03$ ;  $p < 0.05$ , Wilcoxon signed-rank test).

The response of DRG explants to NGF could not be predicted solely on the basis of physical characteristics such as explant size, as both neurite outgrowth and guidance ratio were independent of explant size (Fig. 4A and B), with correlation values of  $r^2 = 0.06$  and  $0.02$ , respectively. The guidance ratio also correlated poorly with neurite outgrowth ( $r^2 = 0.04$ ; Fig. 4C), suggesting that the outgrowth and guidance response of DRG explants to NGF are discrete responses and not co-dependent. Exemplifying this, T7 DRG explants responded to NGF with good neurite outgrowth (OG =  $3.01 \pm 0.25$ ), but were only poorly guided (GR =  $-0.04 \pm 0.03$ ), while L4 DRGs responded to identical gradients with a similar outgrowth measure (OG =  $3.17 \pm 0.27$ ) and good guidance (GR =  $0.22 \pm 0.04$ ; Fig. 4B and C).

#### *TrkA* expression

What could be determining the variable degree of guidance between spinal levels? Since TrkA is the high-affinity receptor responsible for mediating the guidance effects of NGF (Gallo et al., 1997), we first hypothesized that TrkA receptor expression would correlate with the degree of guidance and outgrowth at different spinal levels. We therefore determined TrkA receptor expression for the metameric DRG series at both the mRNA and protein levels (Fig. 5A–D). Neither TrkA mRNA expression as determined by real-time PCR nor TrkA protein levels as determined by Western blot analysis varied significantly with spinal level. While TrkA immunostaining levels did show significant variation with spinal level, this



**FIG. 5.** TrkA mRNA and protein levels in freshly isolated DRGs as a function of spinal level. (A) Representative immunofluorescence image of TrkA-stained DRG explant cryosections used for quantification of TrkA expression. (B) TrkA mRNA expression normalized to  $\beta$ -actin mRNA. TrkA expression did not vary significantly (ANOVA,  $p > 0.05$ ) across spinal levels ( $n = 3$  per spinal level). (C) TrkA immunostaining normalized to DAPI. TrkA immunostaining levels across spinal levels did not vary significantly from average (Mann-Whitney test,  $p > 0.05$ ;  $n = 3$ –12 DRG sections from a minimum of 3 dorsal root ganglia per spinal level). (D) TrkA protein levels normalized to  $\beta$ -actin ( $n = 3$ –5 per spinal level). TrkA protein levels across spinal levels did not vary significantly from average (Mann-Whitney test,  $p > 0.05$ ; scale bar in A = 175  $\mu$ m; DAPI, 4,6-diamino-2-phenylindole; DRG, dorsal root ganglia; TrkA, tropomyosin-related kinase A). (Color image is available online at [www.liebertonline.com/neu](http://www.liebertonline.com/neu)).

was not correlated with variations in levels of either neurite outgrowth or guidance (Table 1).

To exclude the possibility that this lack of correlation arose from differences in TrkA expression that occurred during the culture period, we also assessed TrkA expression by immunohistochemistry, Western blot, and real-time RT-PCR in the metameric DRG series after 48 h of culture with NGF (Fig. 6A–D). While some differences in TrkA expression were observed between freshly isolated DRG explants and DRGs cultured in the presence of NGF for 48 h, correlation of guidance ratio and neurite outgrowth with TrkA expression remained poor (Table 2).

**TABLE 1.** CORRELATION ( $r^2$ ) OF TRKA LEVELS FROM FRESHLY ISOLATED DRG EXPLANTS AND GUIDANCE RATIO OR OUTGROWTH

Assay type $T = 0h$	Guidance ratio (GR)	Outgrowth (OG)
Immunostaining	0.04	0.00
Western blot	0.03	0.11
Real-time PCR	0.04	0.04

TrkA levels as determined by immunostaining, Western blot, and real-time PCR at  $T = 0h$  did not correlate with GR or OG of DRG explants in response to NGF.

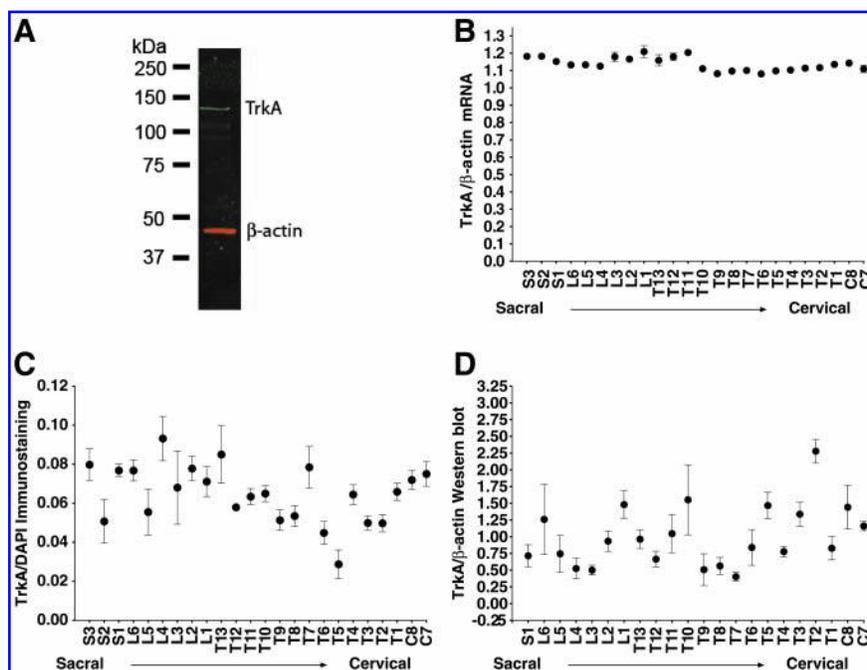
NGF, nerve growth factor; DRG, dorsal root ganglia; TrkA, tropomyosin-related kinase A; PCR, polymerase chain reaction.

## Discussion

We describe for the first time the neurite outgrowth and guidance response of DRGs to identical NGF gradients as a function of rostrocaudal origin of DRG explants. Unexpectedly, the neurite outgrowth response was not consistent across spinal levels, but rather peaked at approximately L6–T7. This was not due to differences in explant size, as the largest explants occurred at level L4, and size declined sharply in adjacent spinal levels. Similarly, the guidance response of DRGs to gradients of NGF was not uniform, with those at L2 and L4 showing above-average guidance, and those at T8 showing below-average guidance. Developmentally, cervical DRGs are formed first (Teillet et al., 1987), so that an increased capacity for regeneration or guidance could be expected for more sacral DRGs. However, the outgrowth response of sacral DRGs was decreased compared to more lumbar location, suggesting that these differences did not arise developmentally.

TrkA is the high-affinity receptor mediating both the outgrowth and turning response of DRG neurons to NGF (Gallo et al., 1997). Surprisingly, we found little correlation of TrkA receptor expression with either neurite outgrowth or guidance ratio, in both freshly isolated DRG explants, and explants cultured for 48 h in the presence of NGF.

It is possible that the methods we used are unable to detect small differences in TrkA levels. An alternative hypothesis is that differences in the internal state of neuronal growth cones at different spinal levels contribute to altered guidance and



**FIG. 6.** TrkA mRNA and protein levels in DRG explants cultured for 48 h in the presence of NGF as a function of spinal level. (A) Representative Western blot used for quantification of TrkA expression (green, TrkA; red,  $\beta$ -actin; ladder, molecular weight marker). (B) TrkA mRNA expression normalized to  $\beta$ -actin mRNA as a function of spinal level. (C) TrkA immunostaining normalized to DAPI as a function of spinal level. (D) TrkA protein levels, normalized to  $\beta$ -actin ( $n = 3-5$  per spinal level; NGF, nerve growth factor; DRG, dorsal root ganglia; TrkA, tropomyosin-related kinase A; DAPI, 4,6-diamino-2-phenylindole). (Color image is available online at [www.liebertonline.com/neu](http://www.liebertonline.com/neu)).

regeneration responses to NGF. Specifically, cAMP levels and PKA activity are able to convert attraction of neurites to neurotrophic guidance cues such as BDNF and NGF to repulsion, and inhibition of PKA reverses the inhibitory effect of NGF on *Sema3a*-mediated growth cone collapse in DRG neurons (Dontchev and Letourneau, 2003; Song and Poo, 1999). Similarly, PKG activity is involved in *Sema3a*-mediated growth cone collapse of DRG neurons, while PKC activity is involved in neogenin signal transduction (Conrad et al., 2007). In addition, RhoA kinase activity (Loudon et al., 2006), as well as cellular calcium levels (Gomez and Zheng, 2006), and membrane potential (McFarlane, 2000) are known to modulate guidance responses to neurotrophic factors. It remains to be determined to what extent any regional differences in these or other downstream signaling pathways may contribute to the response of DRG neurons to NGF.

**TABLE 2.** CORRELATION ( $r^2$ ) OF TRKA LEVELS FROM DRG EXPLANTS CULTURED IN THE PRESENCE OF NGF FOR 48 HOURS AND GUIDANCE RATIO OR OUTGROWTH

Assay type $T = 48$ h	Guidance ratio (GR)	Outgrowth (OG)
Immunostaining	0.05	0.03
Western blot	0.02	0.11
Real-time PCR	0.05	0.22

TrkA levels as determined by immunostaining, Western blot, and real-time PCR at  $T = 48$  h, did not correlate with GR or OG of DRG explants in response to NGF.

NGF, nerve growth factor; DRG, dorsal root ganglia; TrkA, tropomyosin-related kinase A; PCR, polymerase chain reaction.

The findings reported here have clear implications for the study of peripheral nerve regeneration in response to exogenous neurotrophins such as NGF, and also provide insight when assessing the use of NGF in neonatal nerve injuries. Lumbar sites are frequently chosen in animal models of peripheral nerve injury, as functional studies assessing hindlimb innervation are easily carried out. However, this approach may lead to overestimation of the therapeutic potential of exogenous NGF, as both outgrowth and guidance responses are relatively high in DRGs originating from lumbar locations (Fig. 3B and C). While a proportion of peripheral nerve injury occurs in neonates due to brachial plexus and iatrogenic injuries, it remains to be determined if rostrocaudal differences in peripheral nerve regeneration also exist for adult neurons.

While NGF is a relatively well understood model system to study peripheral nerve regeneration and guidance, DRG neurons also respond to many other guidance factors. Specifically, guidance cues used to affect peripheral nerve regeneration include *Sema3A* (Dontchev and Letourneau, 2003; Tang et al., 2007), and GDNF (Mills et al., 2007), as well as BDNF, NT3, FGF-1, and FGF-2 in nerve conduits (Haastert et al., 2008; Midha et al., 2003; Ziemba et al., 2008). In addition, DRG neurons have been shown to be guided by netrin-1 (Masuda et al., 2009), myelin-associated glycoprotein (MAG; Yamashita et al., 2002), and repulsive guidance molecular A (RGMA; Conrad et al., 2007). Therefore, it remains to be determined if other neurotrophins or guidance cues of interest in peripheral nerve regeneration may similarly be subject to variations in effect based on the rostrocaudal origin of the damaged neurons.

## Acknowledgments

This work was supported by grants from the Australian Research Council (DP0666126), the Australian Mental Health and Medical Research Council (project grant 456003), Australian Mental Health and Medical Research Council (project grants 456003 and 631532) and by the Queensland Brain Institute and School of Mathematics and Physics, The University of Queensland. We thank Dr. Timothy Vaughan for helping with quantitative analysis of TrkA immunofluorescence.

## Author Disclosure Statement

No competing financial interests exist.

## References

- Conrad, S., Genth, H., Hofmann, F., Just, I., and Skutella, T. (2007). Neogenin-RGMA signaling at the growth cone is bone morphogenetic protein-independent and involves RhoA, ROCK, and PKC. *J. Biol. Chem.* 282, 16423–16433.
- Crank, J. (1975). *The Mathematics of Diffusion*. 2nd ed. Clarendon Press: Oxford.
- Dontchev, V.D., and Letourneau, P.C. (2003). Growth cones integrate signaling from multiple guidance cues. *J. Histochem. Cytochem.* 51, 435–444.
- Gallo, G., Lefcort, F.B., and Letourneau, P.C. (1997). The trkA receptor mediates growth cone turning toward a localized source of nerve growth factor. *J. Neurosci.* 17, 5445–5454.
- Goldstein, R.S., Avivi, C., and Geffen, R. (1995). Initial axial level-dependent differences in size of avian dorsal root ganglia are imposed by the sclerotome. *Dev. Biol.* 168, 214–222.
- Gomez, T.M., and Zheng, J.Q. (2006). The molecular basis for calcium-dependent axon pathfinding. *Nat. Rev. Neurosci.* 7, 115–125.
- Gundersen, R.W., and Barrett, J.N. (1979). Neuronal chemotaxis: chick dorsal-root axons turn toward high concentrations of nerve growth factor. *Science* 206, 1079–1080.
- Haastert, K., Ying, Z., Grothe, C. and Gomez-Pinilla, F. (2008). The effects of FGF-2 gene therapy combined with voluntary exercise on axonal regeneration across peripheral nerve gaps. *Neurosci. Lett.* 443, 179–183.
- Hamburger, V. and Levi-Montalcini, R. (1949). Proliferation, differentiation and degeneration in the spinal ganglia of the chick embryo under normal and experimental conditions. *J. Exp. Zool.* 111, 457–501.
- Letourneau, P.C. (1978). Chemotactic response of nerve fiber elongation to nerve growth factor. *Dev. Biol.* 66, 183–196.
- Loudon, R.P., Silver, L.D., Yee, H.F. Jr., and Gallo, G. (2006). RhoA-kinase and myosin II are required for the maintenance of growth cone polarity and guidance by nerve growth factor. *J. Neurobiol.* 66, 847–867.
- Masuda, T., Yaginuma, H., Sakuma, C., and Ono, K. (2009). Netrin-1 signaling for sensory axons: Involvement in sensory axonal development and regeneration. *Cell Adh. Migr.* 3, 171–173.
- McFarlane, S. (2000). Attraction vs. repulsion: the growth cone decides. *Biochem. Cell Biol.* 78, 563–568.
- Midha, R., Munro, C.A., Dalton, P.D., Tator, C.H., and Shoichet, M.S. (2003). Growth factor enhancement of peripheral nerve regeneration through a novel synthetic hydrogel tube. *J. Neurosurg.* 99, 555–565.
- Mills, C.D., Allchorne, A.J., Griffin, R.S., Woolf, C.J., and Costigan, M. (2007). GDNF selectively promotes regeneration of injury-primed sensory neurons in the lesioned spinal cord. *Mol. Cell Neurosci.* 36, 185–194.
- Moore, K., MacSween, M., and Shoichet, M. (2006). Immobilized concentration gradients of neurotrophic factors guide neurite outgrowth of primary neurons in macroporous scaffolds. *Tissue Eng.* 12, 267–278.
- Mortimer, D., Feldner, J., Vaughan, T., Vetter, I., Pujic, Z., Rosoff, W.J., Burrage, K., Dayan, P., Richards, L.J., and Goodhill, G.J. (2009). Bayesian model predicts the response of axons to molecular gradients. *Proc. Natl. Acad. Sci. USA* 106, 10296–10301.
- Mortimer, D., Pujic, Z., Vaughan, T., Thompson, A.W., Feldner, J., Vetter, I. and Goodhill, G.J. (2010). Axon guidance by growth-rate modulation. *Proc. Natl. Acad. Sci. USA* 107, 5202–5207.
- Pujic, Z., Mortimer, D., Feldner, J., and Goodhill, G.J. (2009). Assays for eukaryotic cell chemotaxis. *Comb. Chem. High Throughput Screen.* 12, 580–588.
- Rosoff, W.J., McAllister, R., Esrick, M.A., Goodhill, G.J., and Urbach, J.S. (2005). Generating controlled molecular gradients in 3D gels. *Biotechnol. Bioeng.* 91, 754–759.
- Rosoff, W.J., McAllister, R.G., Goodhill, G.J., and Urbach, J.S. (2009). Quantitative studies of neuronal chemotaxis in 3D. *Methods Mol. Biol.* 571, 239–254.
- Rosoff, W.J., Urbach, J.S., Esrick, M.A., McAllister, R.G., Richards, L.J., and Goodhill, G.J. (2004). A new chemotaxis assay shows the extreme sensitivity of axons to molecular gradients. *Nat. Neurosci.* 7, 678–682.
- Savignat, M., Vodouhe, C., Ackermann, A., Haikel, Y., Laval, P., and Libersa, P. (2008). Evaluation of early nerve regeneration using a polymeric membrane functionalized with nerve growth factor (NGF) after a crush lesion of the rat mental nerve. *J. Oral Maxillofac. Surg.* 66, 711–717.
- Song, H.J., and Poo, M.M. (1999). Signal transduction underlying growth cone guidance by diffusible factors. *Curr. Opin. Neurobiol.* 9, 355–363.
- Tang, X.Q., Heron, P., Mashburn, C., and Smith, G.M. (2007). Targeting sensory axon regeneration in adult spinal cord. *J. Neurosci.* 27, 6068–6078.
- Teillet, M.A., Kalcheim, C., and Le Douarin, N.M. (1987). Formation of the dorsal root ganglia in the avian embryo: segmental origin and migratory behavior of neural crest progenitor cells. *Dev. Biol.* 120, 329–347.
- Verge, V.M., Gratto, K.A., Karchewski, L.A., and Richardson, P.M. (1996). Neurotrophins and nerve injury in the adult. *Philos. Trans. R Soc. Lond. B Biol. Sci.* 351, 423–430.
- Yamashita, T., Higuchi, H., and Tohyama, M. (2002). The p75 receptor transduces the signal from myelin-associated glycoprotein to Rho. *J. Cell Biol.* 157, 565–570.
- Ziembra, K.S., Chaudhry, N., Rabchevsky, A.G., Jin, Y., and Smith, G.M. (2008). Targeting axon growth from neuronal transplants along preformed guidance pathways in the adult CNS. *J. Neurosci.* 28, 340–348.

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