

Behaviour of DRG Sensory Neurites at the Intact and Injured Adult Rat Dorsal Root Entry Zone: Postnatal Neurites Become Paralysed, Whilst Injury Improves the Growth of Embryonic Neurites

JON P. GOLDING,^{1*} CHARLES BIRD,² STEPHEN McMAHON,³
AND JAMES COHEN¹

¹Department of Developmental Neurobiology, KCL, Guy's Campus, London, United Kingdom

²Department of Anatomy and Cell Biology, KCL, Guy's Campus, London, United Kingdom

³Division of Physiology, KCL, St. Thomas' Campus, London, United Kingdom

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ABSTRACT The dorsal root entry zone is a PNS-CNS junction between Schwann cells and astrocytes, defining the site where dorsal root ganglia (DRG) axons enter the adult mammalian spinal cord. Following dorsal root injury (rhizotomy), DRG axons regenerate within the PNS environment of the root but stop at the DREZ and fail to re-enter the spinal cord. We have used an *in vitro* model to compare how neurites growing from embryonic (E13) and postnatal (P0 and adult) DRG neurons behave at the uninjured and rhizotomized adult rat DREZ. We find that both freshly dissected and conditioned-lesioned postnatal DRG neurons seldom grow neurites across cryosections of the uninjured or rhizotomized DREZ. However, embryonic DRG neurons more readily grow neurites across cryosections of the uninjured and 7-day post-lesion (dpl) DREZ and are dramatically better able to cross the 21 dpl DREZ. This enhanced growth was abolished by co-incubation with a function-blocking antiserum to $\beta 1$ -integrin receptors, whilst immunoreactivity for some $\beta 1$ -integrin ligands (tenascin-C and fibronectin) increased at the DREZ by 21 dpl, suggesting that $\beta 1$ -integrin ligands may stimulate the growth of embryonic neurites across the 21 dpl DREZ. Fluorescence time-lapse video-microscopy was used to record the behaviour of dye-labelled postnatal DRG neurites as they encounter the uninjured adult DREZ *in vitro*. Neurites rarely turned around at the DREZ, but instead became paralysed. Of a variety of chemical modifications to uninjured DREZ cryosections, only treatment with methanol, chloroform, or the protease inhibitor D-phe-pro-arg chloromethylketone hydrochloride (PPACK, 100 μ M) caused any increase in the proportion of postnatal neurites which crossed the DREZ. *GLIA* 26:309–323, 1999. © 1999 Wiley-Liss, Inc.

INTRODUCTION

Primary sensory neurons of the dorsal root ganglia (DRG) project axons both to the periphery and, via the dorsal roots (DR), to the spinal cord. At the junction between the DR and the spinal cord in mature mam-

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Jon P. Golding is currently at the Division of Neurobiology, National Institute for Medical Research, The Ridgeway, Mill Hill, London NW7 1AA, UK.

*Correspondence to: Dr. J. Golding, Division of Neurobiology, National Institute for Medical Research, The Ridgeway, Mill Hill, London NW7 1AA, United Kingdom. E-mail: jgoldin@nimr.mrc.ac.uk

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mals is a specialised PNS-CNS interface, the dorsal root entry zone (DREZ), where the glial ensheathment of the centrally-projecting DRG afferent axons changes abruptly from that of Schwann cells in the DR to astrocytes in the spinal cord (Berthold and Carlstedt, 1977a; Golding et al., 1997). Following DR crush injury (rhizotomy) in adult mammals, several cellular changes occur between the DR injury site and the synaptic terminals of degenerating DRG axons in the spinal cord. Macrophages invade the DR to phagocytose myelin and axonal debris (Avellino et al., 1995), whilst Schwann cells deprived of axon contact revert to a non-myelin forming phenotype (Avellino et al., 1995) and express the p75 low-affinity neurotrophin receptor (Gai et al., 1996). Within the spinal cord, and especially at the DREZ, astrocytes undergo "reactive" changes which include an increased expression of the intermediate filament-associated protein GFAP and extension of astrocytic processes further distally into the DR, between the endoneurial tubes (Bignami et al., 1984). Although injured DRG axons are able to successfully re-grow through the PNS environment of the DR, they stop when they encounter reactive astrocytes at the rhizotomized DREZ and thus fail to reconnect with the spinal cord, resulting in the permanent loss of sensation and motor control in the affected periphery (Cajal, 1928; Carlstedt, 1985; Stensaas, 1987). This has led to the hypothesis that reactive astrocytes at the DREZ may present a molecular barrier to regenerating DRG axons. In support of this idea, elevated concentrations of tenascin and certain sulphated proteoglycans, molecules which repel growing DRG axons which encounter them as substrate boundaries in vitro (Snow et al., 1990; Taylor et al., 1993), are found in association with reactive astrocytes within the injured dorsal columns and at the DREZ following rhizotomy (Pindzola et al., 1993).

In contrast to the idea of an up-regulation of axon growth inhibitory molecules in response to DREZ injury, other data suggests that developmental changes may be primarily responsible for generating a constitutive barrier to regenerating axons at the DREZ. Thus, in vivo studies have shown that the rat DREZ changes from a conduit for DRG axon growth into a barrier to regenerating DRG axons by the end of the first postnatal week (Carlstedt, 1988), although at this young age reactive changes are not apparent at the rhizotomized DREZ (Pindzola et al., 1993). Furthermore, an in vitro model of the rat DREZ, in which dissociated DRG neurons were seeded onto longitudinal cryosections of *uninjured* 1-week-old or adult spinal cord, demonstrated that the majority (about 95%) of growing postnatal DRG neurites fail to cross the DREZ, in the absence of any gliosis (Golding et al., 1996). In the same experimental paradigm, when early embryonic DRG neurons were seeded onto uninjured one-week-old spinal cord cryosections, up to 35% of neurites which encountered the DREZ were able to extend neurites across it onto the spinal cord (Golding et al., 1996), suggesting that functional neuronal receptors for DREZ

inhibitory ligand(s) are only acquired during the latter stages of embryonic development.

The DREZ inhibitory activity and its mechanism of action are currently unidentified. However, in other regions of the developing and maturing nervous system, axon growth inhibitors can be broadly grouped into two classes on the basis of their effects on axonal growth cones. The first group cause long-term paralysis of growth cones and include: agrin (Campagna et al., 1995), ephrin-A5 (formerly known as RAGS/AL-1) (Drescher et al., 1995; Meima et al., 1997a), and Oligodendrocyte cell surfaces (Bandtlow et al., 1990). The second group cause short-term growth cone or filopodial collapse/retraction, allowing axons to turn and grow away from the repulsive molecule without a significant pause and these include: certain chondroitin sulphated proteoglycans (Snow et al., 1990, 1991), collapsin (Luo et al., 1993; Fan and Raper, 1995), tenascin (Taylor et al., 1993), the proteinase Erase (Baird and Raper, 1995), T-cadherin (Fredette et al., 1996), myelin-associated glycoprotein (Li et al., 1996), and ephrin-B1 (formerly known as Lerk2) (Meima et al., 1997b).

In the present study we have used our cryoculture model of the rat DREZ to show that growing embryonic and postnatal DRG neurites behave differently when they encounter either the uninjured or pre-lesioned adult rat DREZ in vitro. In addition, we have made chemical modifications to the DREZ and used time-lapse video microscopy to begin addressing the mechanisms which prevent postnatal DRG neurites from crossing the adult DREZ.

MATERIALS AND METHODS

Surgery and Preparation of Cryosections

A total of 12 adult Wistar rats were anaesthetised with pentobarbitone (40 mg/kg ip). The cauda equina was exposed by dorsal laminectomy and the dorsal roots (DR) S1, S2, and L1 were completely transected on the right side of the body approximately 1 cm from their junction with the spinal cord at the DREZ. After 7 or 21 days post lesion (dpl), the animals were killed by carbon dioxide asphyxiation and the lumbar enlargement of the spinal cord was aseptically removed and mounted longitudinally on cork mats with the dorsal surface uppermost and snap frozen in liquid nitrogen, as described previously (Golding et al., 1996). In addition, the lumbar enlargement of the spinal cord was aseptically removed from six uninjured adult Wistar rats, mounted on cork mats, and frozen as described above. Lumbar DR were also dissected from five uninjured adult Wistar rats, cut into 4–5 mm segments, and arranged into parallel rows on thin slices of adult liver on cork mats. Longitudinal 10 μ m cryostat sections were cut, which incorporated the DR and DREZ on both the uninjured and rhizotomized sides of the operated spinal cords, or similar longitudinal 3–5 μ m cryostat sections were cut through the uninjured spinal cords or through the segments of DR. The hemi-rhizotomized sections were picked up on sterile 13 mm diameter

polylysine-coated glass coverslips (BDH, UK), whilst the uninjured spinal cord and DR sections were thawed onto individual polylysine-coated glass-bottomed 35 mm diameter filming dishes (MatTek Corporation, Ashland, MA). All sections were stored in a sterile environment at -70°C for no longer than 2 weeks.

For the preparation of conditioned lesion DRG neurons a total of four adult Wistar rats were anaesthetised, as detailed above, and the sciatic nerve was transected on the right side of the body. Seven days later the animals were killed and the denervated DRGs were aseptically removed and dissociated, as described below.

Preparation of Dissociated DRG Neurons and Culture on Cryosections

This was performed as described previously (Golding et al., 1996). Briefly, the DRGs were aseptically dissected from uninjured E13, newborn (P0) and adult rats and also from conditioned lesion adult rats and were collected in equilibrated Ham's F12 medium (Gibco, UK) where the attached roots were trimmed away. Embryonic and P0 DRGs were enzymatically treated with 0.5 ml of 10 U/ml papain (Lorne Laboratories, UK) in HEPES-buffered saline, containing 0.4 mg/ml cysteine (Sigma, UK) at 37°C for 10–20 min. Adult DRGs were enzymatically treated with 0.5 ml of 0.125% collagenase (Sigma, UK) in F12 medium for 40 min at 37°C . Following enzymatic treatment, all DRGs were washed with three changes of F12 and were then briefly triturated in 100 μl of $\text{Ca}^{2+}/\text{Mg}^{2+}$ -free Earle's balanced salt solution (Gibco, UK) containing 50 $\mu\text{g}/\text{ml}$ DNase (type I; Sigma, UK). DRG cell suspensions were diluted to give 15,000 cells/ml in Bottenstein and Sato's medium, containing 2% foetal calf serum, NGF (100 ng/ml), NT-3 (10 ng/ml), and BDNF (50 ng/ml) (BSF-2). For each experiment a minimum of four coverslips, each bearing two unfixed cryostat sections, were placed into individual wells of 24-well multidishes (Greiner, UK) and were washed with 1 ml F12 for 20 min. This solution was then replaced with 0.5 ml of DRG cell suspension and cultures were maintained at 37°C in a humidified atmosphere of 5% CO_2 and 95% air for 18 h prior to fixation and immunostaining.

Dye-Labeling of Neurons for Time-Lapse Microscopy

Dissociated P0 DRGs were used for fluorescence time-lapse studies and these were incubated for 50 min at 37°C with 1 $\mu\text{g}/\text{ml}$ CMFDA 'Cell Tracker' dye (5-chloromethylfluorescein diacetate, Molecular Probes, Portland, OR) at a density of approximately 80,000 cells/ml in BSF-2. Labelled DRGs were then centrifuged at 200g for 5 min, the supernatant was removed and cells were spin-washed with 2×10 ml changes of F12, before being finally re-suspended in 1ml BSF-2. Prior to applying dye-labelled DRG cell suspension to

spinal cord cryosections in glass-bottomed filming dishes, the unfixed cryosections were immunostained with a rabbit anti-laminin-1 antiserum (1:800 in BSF-2; Sigma, UK) for 1 h at room temperature, followed by a series of washes in BSF-2. AMCA-conjugated goat anti-rabbit secondary antibody (1:200 in BSF-2; Molecular Probes, Portland, OR), pre-absorbed against rat dorsal roots, was then applied for 30 min, followed by another series of washes in BSF-2. Laminin immunostaining delineated the boundary between the laminin-rich DR and the spinal cord at the DREZ and in preliminary experiments was not found to affect neurite growth on the DR.

Two hundred microlitres of DRG cell suspension was plated onto cryosections of adult spinal cord or DR in glass-bottomed filming dishes at a density of 20,000 cells/ml. For time-lapse recording of unlabelled and CMFDA dye-labelled neurites, growing on laminin-coated substrates, 200 μl of DRG cell suspension was plated at a density of 10,000 cells/ml onto glass-bottomed filming dishes, which had previously been incubated with 100 $\mu\text{g}/\text{ml}$ laminin for 3 h and then washed with BSF-2. DRGs were allowed to settle and attach to the cryosection or laminin-coated substrate for 2 h at 37°C in a humidified atmosphere of 5% CO_2 and were then supplemented with a further 4 ml of BSF-2 medium, buffered with 15 mM HEPES. Cultures were then transferred to the inverted-stage of a Nikon Optiphot-2 epi-fluorescence microscope, which was enclosed in a humidified atmosphere at 36°C throughout the subsequent time-lapse recordings.

Time-Lapse Video-Microscopy

Cultures were maintained on the stage of a Nikon Optiphot-2 inverted-stage epi-fluorescence microscope, as described. Unlabelled neurites were continuously illuminated with low intensity white light from a tungsten bulb, whilst dye-labelled neurites were illuminated with brief (5 ms) exposures to a quartz/halogen light source, equipped with FITC and AMCA filter sets and neutral density filters to reduce the incident beam intensity. AMCA-stained laminin immunoreactivity was used to delineate the PNS-CNS junction of the DREZ, whilst FITC illumination was used to visualise CMFDA dye-labelled neurons and growing neurites. The timing of the light exposures was computer controlled and used a high speed shutter (UniBlitz, Vincent Associates, UK), which was synchronised with the image acquisition system. Care was taken to ensure that the neuron cell body was not directly illuminated, since in preliminary studies this often caused neuronal cell death.

Digitised images were captured every 10 min over the course of 4–12 h using a low light level CCD camera (IC-100, Photon Technology International, UK), linked to a digital framegrabber (DT2876, Data Translation, Marlboro, MA). In each experiment, the first image to be acquired was of the AMCA-stained laminin immunoreactivity, whilst subsequent images were of CMFDA-

labelled neurites and were illuminated with the FITC filter set. A second image of the laminin immunoreactivity was captured at the end of each experiment and compared with the first image to confirm that there had been no movement of the dish during the filming period. To quantify neurite movement, the pixel co-ordinates of the leading edge of the neurite growth cone were measured in successive digitised images, and it was assumed that neurites grew along the shortest path between each measured point. This gave the neurite growth rate from frame to frame in units of pixels/10 min, and a calibration slide was used to convert pixel distances into microns. Growth rates were then expressed in $\mu\text{m/hr}$. In order to better illustrate the interactions between growing neurites and the DREZ, several of the time-lapse sequences shown are false-coloured composite images of CMFDA dye-labelled DRG neurons and the AMCA anti-laminin immunostained DREZ substrate.

Immunohistochemistry

Cultures were fixed with 2% paraformaldehyde in PBS for 10 min, permeabilized with 100% cold methanol for 3 min, and then washed with PBS. Cultures were then dual immunostained for 1 h in antibody diluent (10% FCS in MEM containing 15 mM HEPES, pH 7.4), containing a guinea pig antiserum which recognises rat laminin-1 and laminin-2 (1:200; Golding et al., 1997), to identify the basal lamina of DR endoneurial tubes; and a rabbit anti-GAP-43 antiserum (1:1,000; a gift of Dr. Graham Wilkin, Imperial College, London), to identify cultured DRG neurons and their growing neurites. Cultures were washed in three changes of PBS and were then incubated for 1 h in diluent containing a mixture of Cy3-conjugated goat anti-rabbit-Ig (1:200; Amersham, UK) and fluorescein-conjugated goat anti guinea pig-Ig (1:200; Chemicon, UK) secondary antibodies.

Some cryosections were immunostained in the absence of any applied DRG neurons. They were fixed with 2% paraformaldehyde in PBS, washed in PBS, and blocked in diluent for 2 h. Pairs of antibodies in diluent were then used to dual immunostain these sections. These antibody pairs always included guinea pig anti-laminin (1:200) and either rabbit anti-laminin-1 (1:200; Sigma, UK), rabbit anti-tenascin-C (PK7, 1:100; a gift of Prof. Melitta Schachner, Centre for Molecular Biology, University of Hamburg, Germany), rabbit anti-fibronectin (28XYZ, 1:100; a gift of Dr. Richard Hynes, Center for Cancer Research, Howard Hughes Medical Institute, MIT, MA), rabbit anti-low molecular weight neurofilament (1:500; a gift of Dr. Peter Hollenbeck, Department of Neurobiology, Harvard Medical School, Boston, MA), monoclonal mouse anti-p75 neurotrophin receptor (clone 192, 1:100; Boehringer, UK), or monoclonal mouse anti-GFAP (clone G-A-5, 1:500; Sigma, UK). Sections were washed in PBS and then incubated with fluorescein-conjugated goat anti-guinea pig-Ig (1:200) and either Cy3-conjugated goat anti-rabbit-Ig (1:200) or Cy3-conjugated goat anti-mouse-Ig (1:200; Amersham,

UK). After staining, all sections were post-fixed in methanol for 5 min and mounted in glycerol, containing 2.5% 1,4-diazobicyclo-(2,2,2)-octane anti fade reagent (Sigma, UK).

Modifications of Cryoculture Conditions

Certain pre-treatments of the adult spinal cord cryosections were made, prior to applying dissociated DRG neurons and incubating the cultures overnight. These pre-treatments included incubating the cryosections with either: 50 μl 100 μM D-phe-pro-arg chloromethylketone hydrochloride (PPACK, Calbiochem, La Jolla, CA) in PBS for 2 h at room temperature; 50 μl 1 U/ml PI-PLC (Oxford Glycosystems, UK) in DMEM for 2.5 h at 37°C; 50 μl 0.8 U/ml α -fucosidase (EC 3.2.1.51, Oxford Glycosystems) in the manufacturer-supplied buffer for 5 h at 37°C; 30 μl 2.5 U/ml chondroitinase ABC (EC 4.2.2.4, Oxford Glycosystems) in the manufacturer-supplied buffer for 2.5 hr at 37°C; 0.5 ml 0.5% 3-[(3-chloramidopropyl) dimethylammonio]-1-propanesulphonate (CHAPS, Sigma, UK) in PBS for 1.5 h at room temperature; 1 ml methanol (BDH) for 1 h at room temperature; 1 ml chloroform (BDH) for 1 h at room temperature, followed by air drying; 1 ml methanol (BDH) for 15 min, then 1 ml chloroform (BDH) for 1 hr, then 1 ml methanol for a further 15 min all at room temperature; or 4 \times 1 ml changes of 3 M NaCl over 2 h at

Fig. 1. Antigenic changes at the DREZ following rhizotomy. Dual immunofluorescence images of uninjured control and rhizotomized DREZ, stained with anti-laminin (shown in green) and either anti-GFAP (red staining in A-C) or anti-p75 (red staining in D and E). GFAP immunoreactivity (arrows) steadily increases on the rhizotomized side of the spinal cord and DREZ between 7 dpl and 21 dpl, with GFAP +ve astrocyte processes penetrating deeper into the rhizotomized DR. p75 immunoreactivity labels a small number of cells (arrows) in the uninjured DR, but by 7 dpl becomes dramatically increased throughout the rhizotomized DR. Scale bar = 50 μm .

Fig. 2. Embryonic, but not postnatal DRG neurons, extend neurites more readily across the 21 dpl rhizotomized DREZ in vitro. A-D: Contralateral uninjured (A,C) or rhizotomized (B,D) sides of 21 dpl hemi-rhizotomized spinal cord cryosections, dual immunostained with anti-laminin (green staining of the DR), and anti-GAP-43 antibodies (red staining of applied dissociated DRG neurons and their growing neurites). In A and B, embryonic day 13 (E13) DRG neurons (n) extend neurites along both the contralateral (A) or rhizotomized (B) dorsal root (DR) towards the spinal cord (SC), where they encounter and cross the DREZ (dotted line in A) at the points marked by arrowheads. E13 neurites cross the rhizotomized DREZ more readily than the uninjured DREZ. In C and D, postnatal day zero (P0) DRG neurons (n) extend neurites along the contralateral (C) or rhizotomized (D) DR. These neurites generally stop at the DREZ (at the points marked with arrows) and fail to extend onto the spinal cord. E: Of those neurites which encounter the DREZ, the proportion which cross the DREZ onto the spinal cord was quantified. A higher proportion of E13 neurites cross the 21 dpl rhizotomized DREZ than the uninjured contralateral DREZ. This difference is not apparent when E13 DRG neurons are seeded onto 7 dpl hemi-rhizotomized spinal cord cryosections. Similarly, there is no increase in the proportion of neurites which cross the DREZ when postnatal DRG neurons are seeded onto 7 dpl or 21 dpl hemi-rhizotomized spinal cord cryosections. The number of neurites counted per condition is marked within each bar and the error bars are SEM. Student's *t*-test values are: * = $P < 0.05\%$, ** = $P < 0.01\%$, ND = not determined. F,G: Anti-laminin (green) and anti-GAP-43 (red) dual-immunostained cryocultures of contralateral uninjured (F) and 21 dpl rhizotomized (G) DREZ, seeded with conditioned lesion adult DRG neurons (n). Although conditioned lesion neurons show enhanced neurite outgrowth on the DR, they stop at both the contralateral and rhizotomized DREZ (arrows) in the same manner as neurites growing from non-conditioned adult DRG neurons. Scale bars = 50 μm .

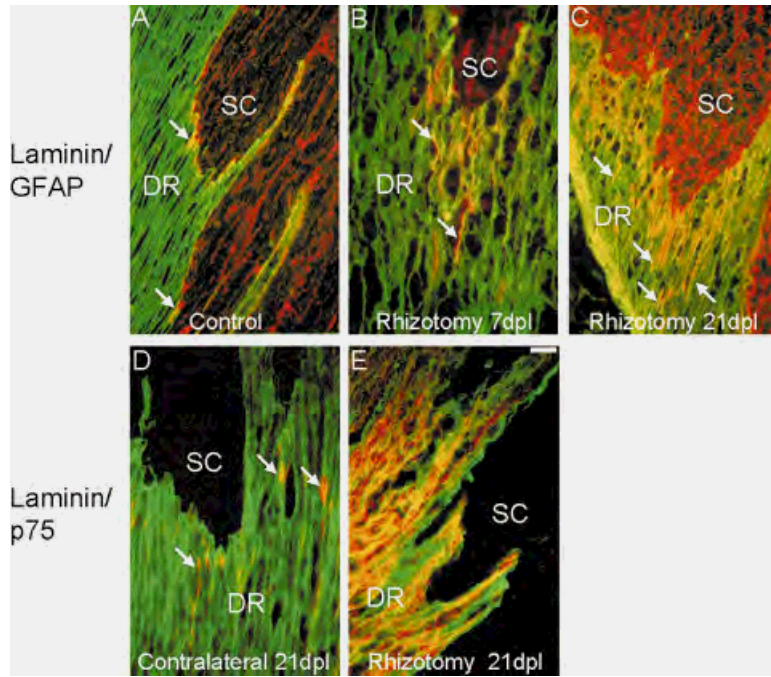


Figure 1.

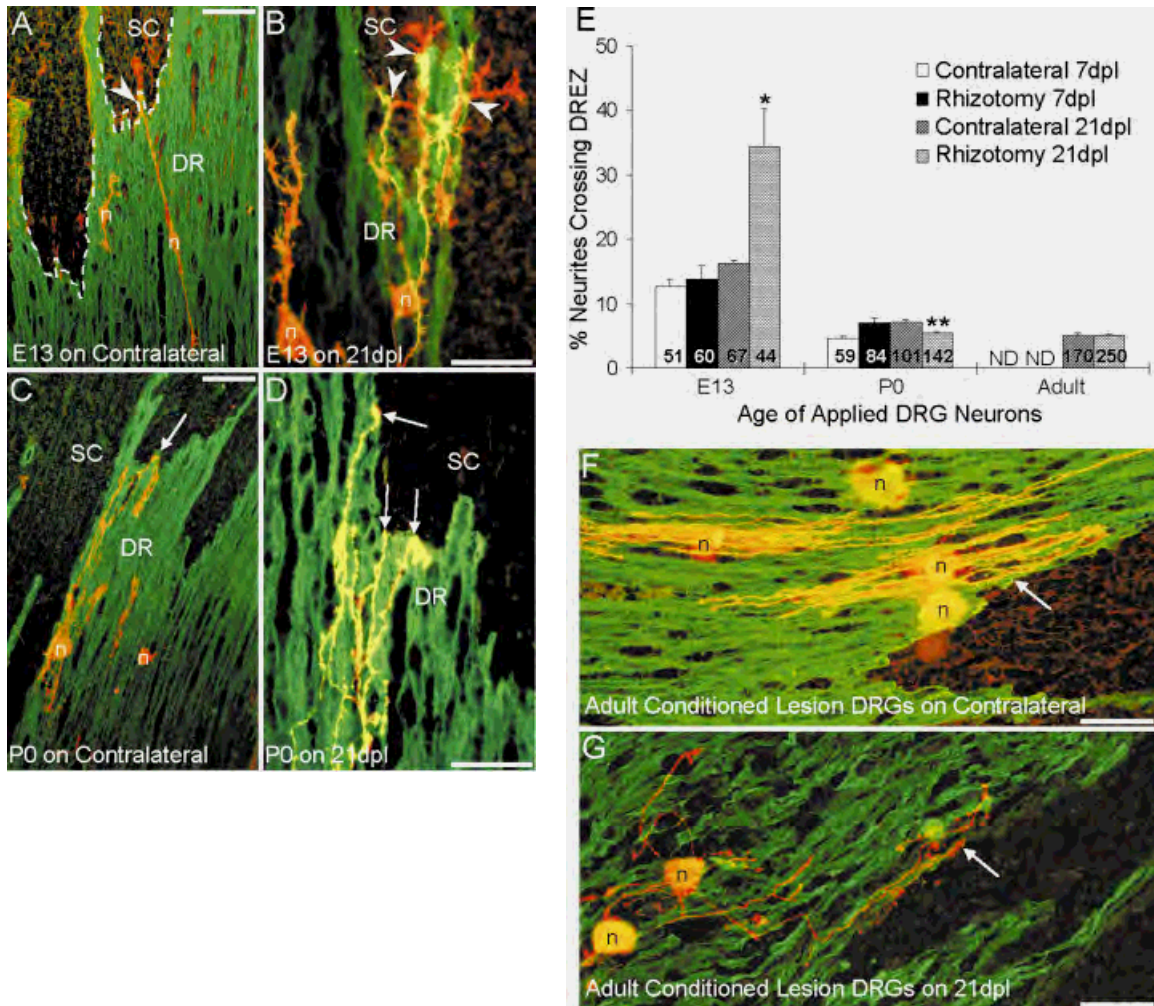


Figure 2.

room temperature. All cryosections were then thoroughly washed with several changes of PBS or DMEM prior to applying DRG neuron cell suspensions and culturing overnight at 37°C in a humidified atmosphere of 5% CO₂. Control experiments with chondroitin sulphate-specific antibodies (CS-56, Sigma or chondroitin-6-sulphate specific antibody, ICN Cat. No. 636521), and fucose-specific lectins (*Aleuria aurantia*, Vector Laboratories and *Lotus tetragonolobus*, Oxford Glycosystems) were used to confirm the efficacy of chondroitinase and fucosidase enzymatic treatments on spinal cord cryosections (not shown). Control experiments with an antibody recognising the GPI-anchored molecule mCD24 were used to confirm that phospholipase-C treatment dramatically reduced immunostaining on cell monolayers (Shewan et al., 1996) and cryosections of tissues expressing high levels of mCD24 (not shown). In some experiments, the culture medium was supplemented with certain drugs or enzymes and the cultures were then maintained overnight at 37°C, as normal. These supplements included either: 0.8 U/ml PI-PLC (Oxford Glycosystems); 300 µM suramin (sodium salt, Calbiochem, La Jolla, CA); or 200 ng/ml pertussis toxin (Sigma, UK). In other experiments the medium was supplemented with either a function-blocking rabbit antiserum to the β1 integrin sub-unit (1:100, 20 µg/ml; a gift of Dr. Kristofer Rubin, Uppsala University, Sweden) or a 1:100 dilution of normal rabbit serum as a control. Cultures were subsequently fixed and dual immunostained with anti-laminin and anti-GAP-43 antisera, as described.

Quantitative Measurements of Neurite Outgrowth

Digitized images of cryocultures, dual-immunostained with guinea pig anti-laminin and rabbit anti-GAP-43 antibodies, were captured using a frame grabber (DT2876, Data Translation) linked to a Nikon Optiphot-2 epi-fluorescence microscope, equipped with a CCD camera (model FA87, Grundig, Fürth, Germany). The lengths of GAP-43 +ve neurites were then measured using an image analysis program (Optimas v3.10, Media Cybernetics, Bothell, WA). Neurites which grew along the DR and encountered the DREZ either stopped or continued growing across the DREZ and onto the spinal cord. Those neurites, which extended by more than 10 µm from the DR onto the spinal cord, were deemed to have crossed the DREZ. The number of crossing events was quantified and expressed as a percentage of the total number of neurite encounters with the DREZ. The length by which each neurite grew onto the spinal cord from the DREZ was quantified. The longest neurite per neuron, growing from DRG neurons that had attached to and were growing exclusively on spinal cord adjacent to the DREZ, was also quantified. All experimental conditions were repeated on three to four separate occasions and the statistical significance of neurite movement rates and turning or crossing events at the DREZ was assessed by Student's *t*-test or

chi-squared contingency tables and values are displayed as the mean value ± SEM.

RESULTS

Cellular Changes at the DREZ Following Rhizotomy

Rhizotomy leads to rapid, well characterised changes in the cellular composition and organisation of the DREZ (Bignami et al, 1984). Thus, dual immunolabelling by using a combination of guinea pig anti-laminin and mouse anti-GFAP antibodies was used to identify and compare these changes on longitudinal sections through the intact (Fig. 1A); early, 7 dpl (Fig. 1B); and late, 21 dpl (Fig. 1C) hemi-rhizotomized adult rat spinal cord. Throughout the spinal cord, GFAP immunoreactivity identified astrocytes, which were always more intensely immunolabelled at the glial limitans and at the DREZ (Fig. 1A). In contrast to the intact DREZ, the intensity of GFAP immunolabelling was greatly increased in the rhizotomized DREZ, further increasing between 7 dpl and 21 dpl, with many astrocyte processes extending into the DR for up to 250 µm (Fig. 1B,C). Laminin immunoreactivity delineated the Schwann cell endoneurial tubes within the roots which were parallel aligned along the uninjured DR (Fig. 1A), in contrast to their disorganised arrangement in the DR at 7 dpl and 21 dpl (Fig. 1B,C).

As well as astrocytic changes at the rhizotomized DREZ, injury-associated changes were also observed within the DR. Thus, cryosections of 21 dpl hemi-rhizotomized spinal cord, dual immunostained with antibodies to laminin and the p75 low affinity neurotrophin receptor, revealed few p75 +ve cells (presumed to be non-myelin forming Schwann cells) within the contralateral DR (Fig. 1D), whilst the rhizotomized DR and DREZ was intensely labelled throughout by anti-p75 antibody (Fig. 1E). Neurofilament antibody staining (not shown) confirmed that regenerating DRG axons were absent from the rhizotomized DR, thereby excluding these as a possible source of the p75 immunoreactivity.

Neurites From Embryonic DRG Neurons Cross the Rhizotomized DREZ More Readily Than the Uninjured DREZ

To assess the influence of rhizotomy on the behaviour of DRG neurites at the DREZ, dissociated DRG neurons, isolated from E13, neonatal (P0), or adult rats, were cultured on cryosections of either 7 dpl or 21 dpl hemi-rhizotomized adult spinal cord. Neurons, which adhered to the injured or contralateral uninjured DR within the cryosections, extended neurites along the endoneurial tubes both towards and away from the spinal cord (e.g., Fig. 2A,C,F). At the DREZ, neurites either stopped abruptly or continued growing and crossed onto the spinal cord (Fig. 2A-G).

When E13 or P0 DRG neurons were seeded onto 7 dpl hemi-rhizotomized spinal cord cryosections, they showed no preferential crossing of the rhizotomized DREZ (Fig. 2E; E13: $13.9 \pm 1.9\%$ crossing rhizotomized DREZ, compared with $12.6 \pm 1.2\%$ crossing contralateral DREZ; 60 and 51 neurites, respectively, each in three experiments; P0: $7.0 \pm 0.9\%$ crossing rhizotomized DREZ, compared with $4.6 \pm 0.4\%$ crossing contralateral DREZ; and 84 and 59 neurites respectively, each in three experiments). However, DRG neurons from E13 rats extended neurites twice as frequently across the 21 dpl rhizotomized DREZ and onto the spinal cord than across the contralateral uninjured DREZ (Fig. 2A,B,E; $34.4 \pm 5.9\%$, 44 neurites in four experiments, compared with $16.2 \pm 0.6\%$, 67 neurites in three experiments; $P < 0.05$). By contrast, P0 DRG neurons demonstrated a small but significant decrease in their ability to extend neurites across the 21 dpl rhizotomized DREZ (Fig. 2C-E; $5.4 \pm 0.3\%$ crossing rhizotomized DREZ, compared with $7.2 \pm 0.3\%$ crossing contralateral DREZ; 142 and 101 neurites respectively, each in four experiments; $P < 0.01$). Adult DRG neurons demonstrated no significant difference in their ability to cross either the 21 dpl rhizotomized or uninjured DREZ (Fig. 2E; $5.1 \pm 1.5\%$, 250 neurites and $5.2 \pm 0.2\%$, 170 neurites respectively, each in four experiments).

To parallel the activated condition of DRG neurons following a rhizotomy in vivo, neurons were isolated from adult rats 7 days after receiving a unilateral conditioning lesion of the sciatic nerve. When cultured on cryosections of 21 dpl hemi-rhizotomized spinal cord, they more rapidly and extensively extended neurites on the DR compared with control adult DRG neurons (compare Fig. 2C,D with Fig. 2F,G) but showed no enhanced ability to grow across the DREZ in spinal cord sections from either rhizotomized or uninjured animals ($7.9 \pm 2.8\%$ crossing the rhizotomized DREZ and $7.7 \pm 1.5\%$ crossing the uninjured DREZ. 110 neurites and 64 neurites, respectively, each in three experiments).

Growth of DRG Neurites on the Spinal Cord

The increased facility with which embryonic neurites crossed the 21 dpl rhizotomized DREZ could be explained either by changes intrinsic to the DREZ or to the adjacent CNS. In order to discriminate between these two possibilities, we quantified two aspects of neurite growth on the spinal cord: 1) the distance by which neurites extended onto the spinal cord after crossing the DREZ and 2) the length of neurites growing from neurons which adhered directly to the CNS. For each age of applied DRG neuron, there was no significant difference between the lengths of neurite extension onto either the uninjured or rhizotomized spinal cord from the DR, although neurite extension declined progressively with increasing age of the applied neurons (Fig. 3A). However, E13 DRG neurons, which adhered directly to the spinal cord, grew significantly longer neurites on the 21 dpl rhizotomized side

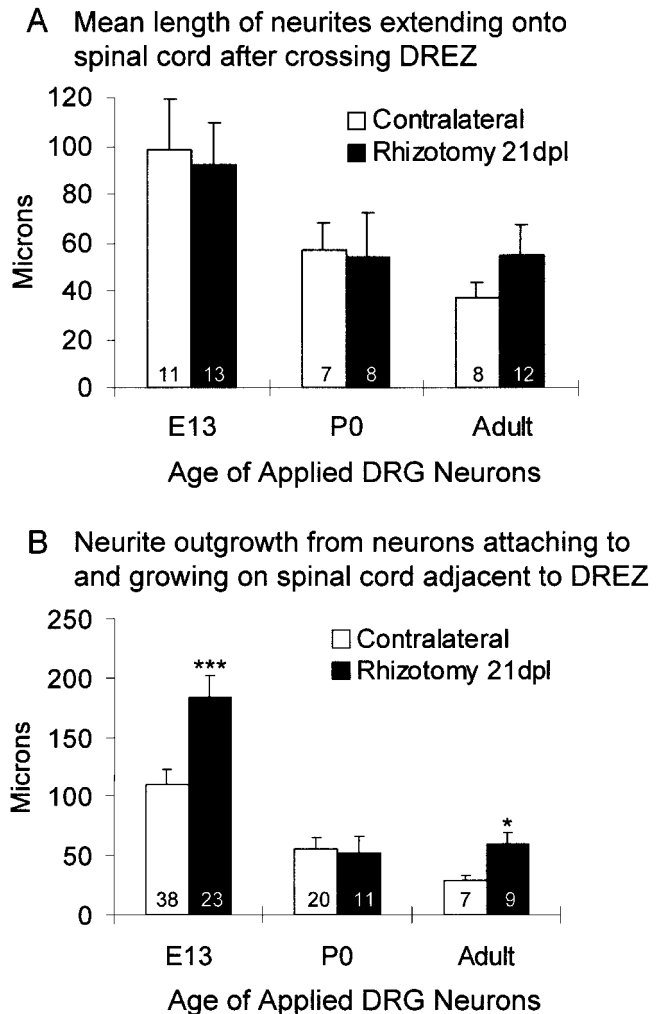


Fig. 3. Neurites grow longer on rhizotomized spinal cord substrates. Bar charts showing the mean length of neurites growing from applied dissociated DRG neurons, isolated from embryonic day 13 (E13), postnatal day zero (P0), and adult rats, cultured on 21 dpl hemi-rhizotomized spinal cord cryosections. **A:** The distance by which neurites cross onto the spinal cord from the DREZ on the contralateral uninjured or rhizotomized sides of the spinal cord. At each age of test neuron examined, neurites which crossed the DREZ extended equally as far onto the uninjured or rhizotomized spinal cord substrate. **B:** However, in the same series of experiments, E13 and adult DRG neurons which adhered directly to the spinal cord substrate grew longer neurites on the rhizotomized side of the spinal cord. The number of neurites counted per condition is marked within each bar and the error bars are SEM. Student's *t*-test values are: * $P < 0.05$, *** $P < 0.001$.

($183.9 \pm 17.9 \mu\text{m}$, 22 neurites) than on the uninjured side ($110 \pm 12.7 \mu\text{m}$, 37 neurites; $P < 0.001$) (Fig. 3B).

$\beta 1$ Integrins and Their ECM Ligands Are Involved in the Growth of Embryonic Neurites Across the Rhizotomized DREZ

Increases both in the extent of growth and the proportion of E13 DRG neurites which cross the 21 dpl rhizotomized DREZ, suggested that they may be responding to a growth-promoting activity associated with the reactive glia at the injured DREZ. Because the

neurite outgrowth promoting activity of several extracellular matrix molecules is mediated by neuronal heterodimeric α - β integrin receptors containing the β 1 sub-unit (Sonnenberg, 1993; Venstrom and Reichardt, 1993), and embryonic rat DRG neurons express many β 1 integrin heterodimers (Tomaselli et al., 1993), we used a polyclonal function-blocking antibody to β 1 integrin to assess whether the increased tendency of neurites from E13 DRG neurons to cross the 21 dpl rhizotomized DREZ involved β 1-containing integrins. The anti- β 1 integrin antibody significantly reduced the proportion of E13 DRG neurites which crossed the 21 dpl rhizotomized DREZ (Fig. 4A, B), whilst it caused a smaller reduction in the proportion of E13 DRG neurites which crossed the uninjured DREZ. Antibody treatment did not have any significant effect on the length by which E13 neurites extended onto the spinal cord after crossing the DREZ, but it did reduce the length of neurites growing from neurons which had attached directly to the spinal cord (Fig. 4B). Antibody treatment also reduced the length of E13 neurite growth on the rhizotomized DR by 70%, from $354.5 \pm 26.4 \mu\text{m}$ (22 neurites) to $150.5 \pm 14.4 \mu\text{m}$ (27 neurites, $P < 0.001$).

Neurite outgrowth promoting molecules that interact with β 1 sub-unit-containing integrin receptors include laminin-1, laminin-2, fibronectin, and tenascin-C (Sonnenberg, 1993; Venstrom and Reichardt, 1993; Varnum-Finney et al., 1995). Immunostaining for these molecules on 7 dpl and 21 dpl hemi-rhizotomized spinal cord cryosections revealed increases in tenascin-C immunoreactivity at the rhizotomized DREZ and increases in fibronectin immunoreactivity throughout the rhizotomized DR and at the DREZ (Fig. 5). These increases were apparent by 7 dpl, but became more pronounced by 21 dpl. By contrast, we detected no changes in laminin-1 or laminin-2 immunoreactivity at either 7 dpl or 21 dpl (not shown).

Neurite Motility Is Dramatically Reduced After Contact With the Uninjured DREZ

The cryoculture technique enabled us to directly observe the behaviour of P0 sensory neurites as they contacted the DREZ, by pre-loading the neurons with CMFDA dye, prior to culture. Initially we determined whether fluorescence illumination of dye-labelled neurites caused any changes in their growth characteristics, compared with phase-contrast illumination. Thus, time-lapse video recordings were made over the course of 4 h of dye-labelled and unlabelled neurites growing from dissociated DRG neurons which had been seeded onto laminin-coated filming dishes. Unlabelled neurites were illuminated with conventional bright-field optics, whilst dye-labelled neurites were exposed intermittently using FITC epi-fluorescence illumination. We found no significant differences in the rate or pattern of neurite outgrowth on the laminin-coated substrate using either source of illumination. Neurites grew at an average rate of $36.3 \pm 4.1 \mu\text{m/hr}$ ($n=6$) under phase-

contrast illumination and at $39.4 \pm 4.6 \mu\text{m/hr}$ ($n=5$) under epi-fluorescence illumination (Fig. 6). Growing neurites demonstrated short bursts of rapid extension, each lasting for about 10–20 min, interspersed by periods of low motility (Fig. 6D).

The behaviour of dye-labelled neurites which approached and interacted with the DREZ from the DR was next studied in cryoculture. Neurites grew along the DR endoneurial tubes in a similar interrupted manner to their behaviour on laminin (compare Figs. 6D, 7C). On contacting the DREZ, growth cones did not collapse, but instead neurite motility fell sharply and within 20 min growth cones became immobile and remained at the DREZ for the duration of the filming period (3–5 h) (Fig. 7).

Neurites might stop growing at the PNS-CNS borderline of the DREZ because of the presence of molecules which actively inhibit neurite motility, or alternatively due to the discontinuation of the neurite outgrowth-promoting laminin-rich DR substrate. To distinguish between these possibilities, P0 neurons were grown on cryosections of isolated DR alone. In these experiments a high proportion of neurites (39.7%, $n=131$) turned around at the cut end of the DR (Fig. 8A), in striking contrast to only 3.7% ($n=103$) of neurites which were observed to turn around at the PNS-CNS borderline of the DREZ ($P < 0.001$, Fig. 8B). Thus, the arrest of growth at the DREZ is consistent with the presence of an inhibitory activity.

Chemical and Enzymic Modifications of the Uninjured DREZ Influence Neurite Growth

We have made a variety of modifications to the spinal cord cryosection substrate or the culture conditions, in an attempt to perturb DREZ barrier properties and thereby characterise the inhibitory factors. Pre-treatment of cryosections with either the irreversible proteinase inhibitor PPACK (Baird and Raper, 1995) (21.5%, $n=69$, $P < 0.01$), methanol (26.2%, $n=167$, $P < 0.001$), chloroform (26.4%, $n=137$, $P < 0.001$), or methanol followed by chloroform (27.7%, $n=167$, $P < 0.001$), all resulted in significant increases in the percentage of P0 neurites which crossed the DREZ. However, we observed no effects after pre-treating cryosections with either the GPI-cleaving enzyme PI-PLC, chondroitinase, fucosidase, the detergent CHAPS, or high concentration salt washes (not shown). Similarly, supplementing the culture medium with either PI-PLC, the heparin analogue suramin, or the G-protein inhibitor pertussis toxin, also failed to affect the proportion of neurites which crossed the DREZ (not shown).

DISCUSSION

A major finding of this study is that neurites growing from embryonic DRG neurons more readily cross the adult DREZ once it has undergone long-term reactive

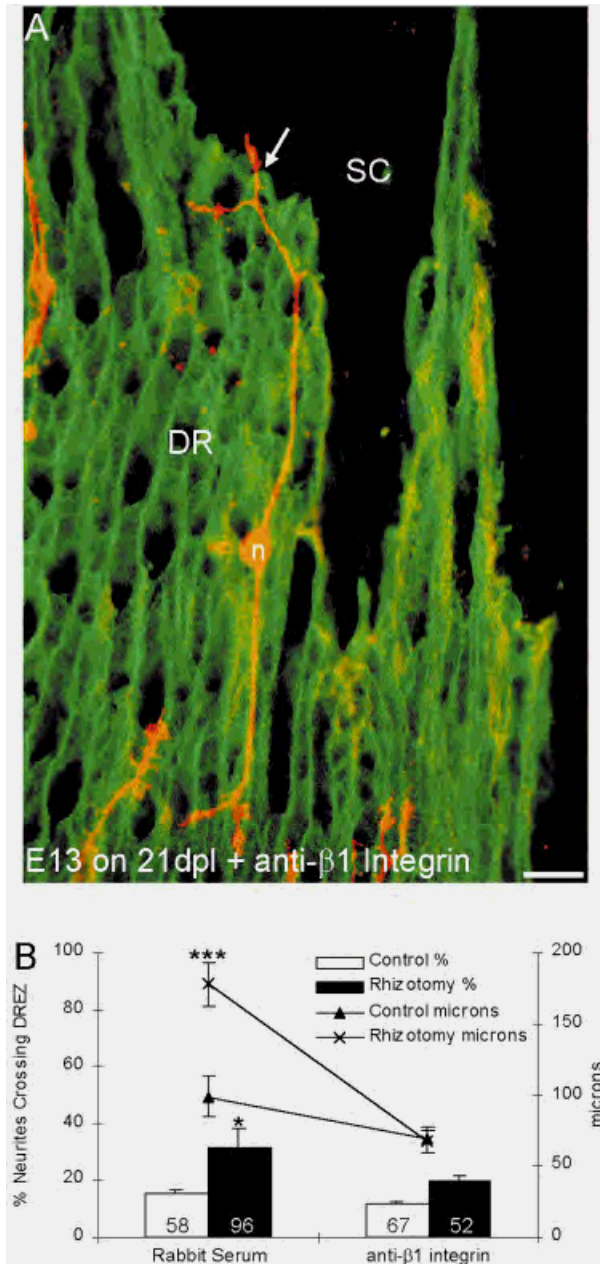


Fig. 4. A: Addition of function-blocking anti-β1 integrin antiserum to cryocultures of embryonic DRG neurons on 21 dpl rhizotomized DREZ reduces the proportion of neurites, which cross the DREZ onto the spinal cord (SC). The dorsal root (DR) is immunostained with anti-laminin antibody (green) and the applied neurons (n) and their growing neurites are immunostained with anti-GAP-43 antibody (red). Neurites can be seen to grow along the DR but stop at the DREZ at the point marked with an arrow. Scale bar = 50 μm. **B:** A bar chart depicting the decrease in the percentage of E13 DRG neurites which cross the DREZ in the presence of anti-β1 integrin antibody, compared with control rabbit serum. The number of neurites counted per condition is marked within each bar. The line chart shows the decrease in the mean length of E13 DRG neurites growing on the spinal cord adjacent to the DREZ in the presence of anti-β1 integrin or rabbit serum. Error bars are SEM. Student's *t*-test values are: **P* < 0.05%, ****P* < 0.001%.

changes after rhizotomy. However, irrespective of its injury status, the DREZ remains a barrier to neurites growing from postnatal DRG neurons. Our data are consistent with the idea that neurite outgrowth promot-

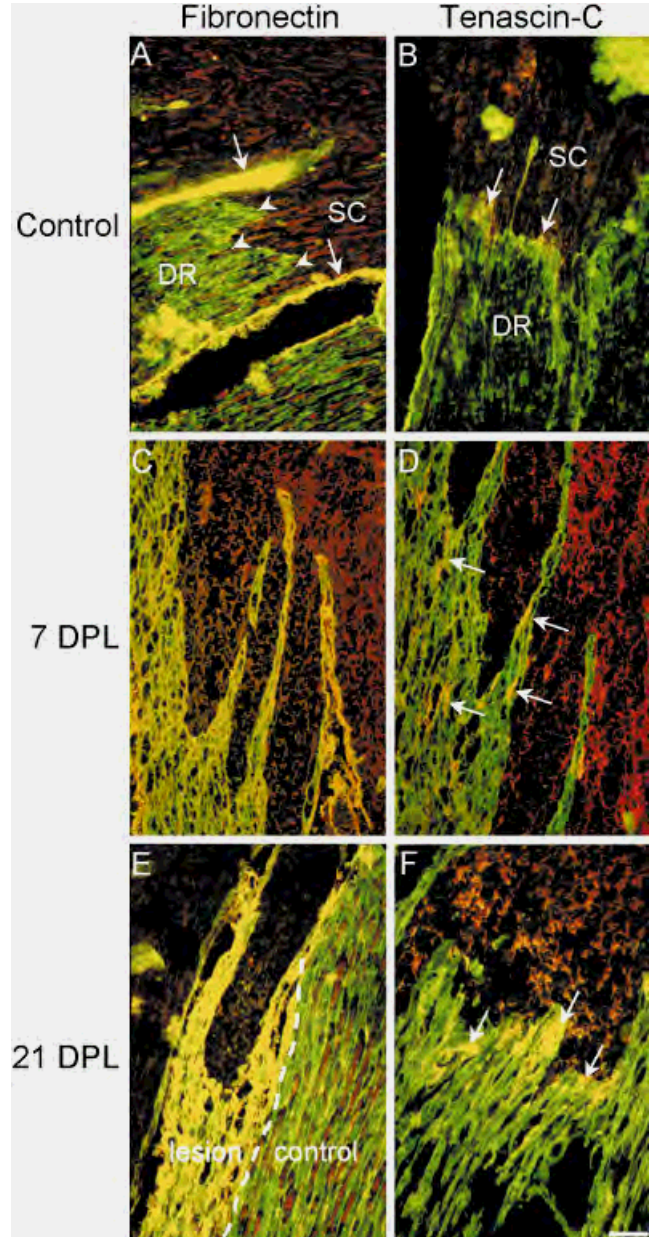


Fig. 5. Some ligands for β1-containing integrins become concentrated at the DREZ following rhizotomy. Control uninjured (A,B), 7 dpl (C,D) and 21 dpl (E,F) DREZ dual immunostained with anti-laminin antibody (green) and either anti-fibronectin (A,C,E) or anti-tenascin-C (B,D,F) antibodies. **A,C,E:** Fibronectin immunoreactivity is predominantly present within the rootlet sheath of the uninjured DR (marked with arrows in A). Following rhizotomy, fibronectin immunoreactivity increases within the DR (C and E). This is most clearly seen in E, where the DR has been partially rhizotomized and the 21 dpl injured (left) side is more strongly immunostained than the uninjured (right) side (a dotted line demarcates the rhizotomized from the uninjured side of the DR). **B,D,F:** Tenascin-C immunoreactivity is present at the PNS-CNS borderline of the uninjured DREZ (marked with arrows in B). By 7 dpl tenascin-C immunoreactivity has increased on the rhizotomized side of the SC and also at the DREZ and within the DR (arrows in D). By 21 dpl there are further increases in tenascin-C immunoreactivity within the SC and especially at the DREZ (arrows in F). Scale bar = 50 μm.

ing molecules become concentrated at the rhizotomized DREZ between 7 dpl and 21 dpl. Because neurites growing from embryonic DRG neurons are less sensi-

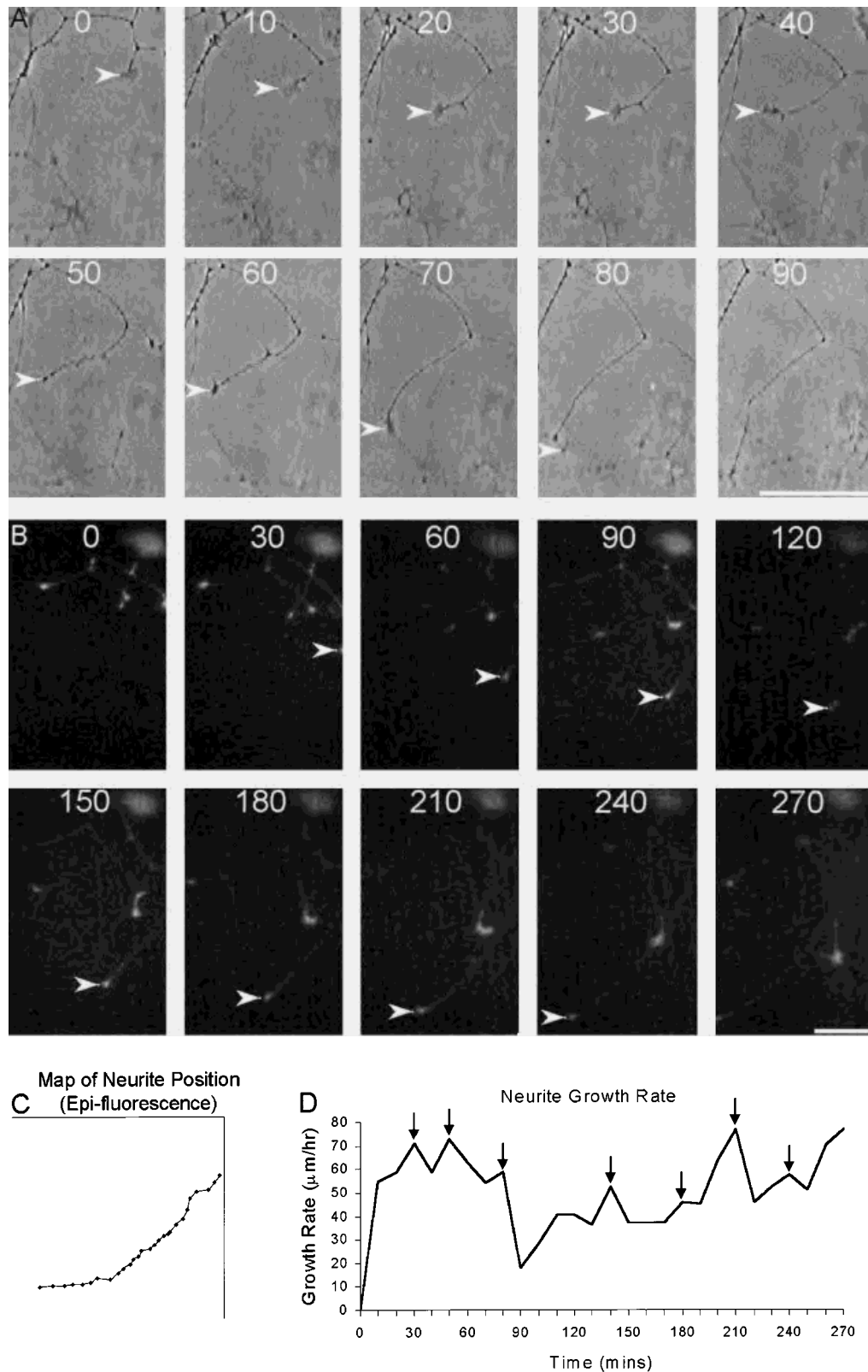


Fig. 6. Comparison between the growth of neurites from unlabelled and CMFDA dye-labelled DRG neurons on laminin-coated substrata, under (A) phase-contrast or (B) epi-fluorescence illumination, respectively. Digitised images of growing neurites (marked with arrowheads in A and B) were captured every 10 min over the course of 4.5 h and the pixel co-ordinates of the growing tips were measured in successive frames (the time in min at which each frame was captured is

indicated). This allowed the calculation of a map of neurite position with time (C) and neurite growth rate (D). (Data sets shown in C and D are for the epi-fluorescence-illuminated time-lapse sequence shown in B.) Neurites grew at the same rate under either source of illumination and exhibited peaks of higher motility, each lasting for about 10 min (arrows in D). Scale bars = 50 μm .

tive to the DREZ barrier than neurites growing from postnatal DRG neurons (Golding et al., 1996), it is only embryonic neurites which can respond to any additional neurite growth-promoting cues at the 21 dpl rhizotomized DREZ. Other studies have similarly shown that embryonic rat DRG neurons are better able than postnatal DRG neurons to grow neurites on CNS tissue cryosections (Shewan et al., 1995) or into three-dimensional astrocyte cultures (Fawcett et al., 1989). Even conditioned-lesioned postnatal DRG neurons, which have a heightened growth capacity (Jacob and McQuarrie, 1993), failed to demonstrate any increased ability to cross the uninjured or rhizotomized DREZ. Therefore, our data indicate that the vigour of neurite growth is not the deciding factor in the ability of neurites to grow across the DREZ, but more likely it is the lack of neuronal receptors for DREZ barrier molecules. Alternatively, the neuronal receptors might be constitutively expressed, but the signal transduction pathway could become modified by cyclic nucleotides, from a growth-promoting signal in embryos to a growth-inhibitory signal in neonates and adults, in an analogous manner to the study by Song et al. (1998).

Besides an increase in neurite growth promoting molecules, we find that additional axon growth inhibitory/repulsive molecules may become concentrated at the rhizotomized DREZ by 21 dpl; since P0 DRG neurons extended fewer neurites across the 21 dpl rhizotomized DREZ than the 7 dpl or uninjured DREZ. Other *in vitro* studies have similarly shown that neurite growth inhibitors become concentrated on long-term reactive astrocytes. Thus, postnatal rodent pontine neurons or DRG neurons grow neurites onto astrocyte monolayers (Wang et al., 1994) or into three-dimensional astrocyte cultures (Fawcett et al., 1989), which have been maintained for less than 10 days but fail to grow into similar cultures which have been maintained for more than 21 days.

Since the enhanced growth of E13 DRG neurites across the 21 dpl rhizotomized DREZ was abolished by anti- $\beta 1$ integrin, and because ligands for $\beta 1$ -containing integrins (tenascin-C and fibronectin) became concentrated at the DREZ between 7 dpl and 21 dpl, it is likely that these or related $\beta 1$ -integrin ligands stimulate embryonic neurite growth across the DREZ. A previous study in the rat (Pindzola et al., 1993) similarly found that tenascin-C immunoreactivity increased within the DR and at the DREZ by 15 days following rhizotomy (but see Zhang et al., 1995), whilst fibronectin expression has been shown to be up-regulated (possibly by Schwann cells) within endoneurial tubes following sciatic nerve injury in rats (Mathews and Ffrench-Constant, 1995). Embryonic rat DRG neurons are known to express the $\beta 1$ -integrin sub-unit (Tomaselli et al., 1993), whilst the growth of chicken DRG axons on tenascin-C and through fibronectin-rich degenerating peripheral nerve has been shown to be mediated by $\alpha 8\beta 1$ and possibly $\alpha 5\beta 1$ integrin receptors, respectively (Varnum-Finney et al., 1995; Lefcort et al., 1992). Furthermore, because splice variants of tenascin-C

(presumably requiring different receptors) can act either as barriers or stimuli to neurite growth *in vitro* (Meiners and Geller, 1997), tenascin-C could simultaneously act both to stimulate embryonic DRG neurite growth and inhibit postnatal DRG neurite growth across the DREZ.

Previous studies have only provided "snap-shots" of the encounters that growing axons make with the DREZ and have left unanswered basic questions concerning how the DREZ provides a barrier to the growth cones of approaching axons: do growth cones collapse, attempt to turn around, or become paralysed after contacting the DREZ? In this study we have addressed this point by recording the dynamic interactions, which postnatal DRG neurites make with the uninjured adult DREZ. We provide the first direct evidence that the failure of these sensory neurites to grow across the DREZ is due to a growth inhibitory activity concentrated in this region, rather than a repulsive activity or passive avoidance of the DREZ by a preference for DR over spinal cord substrates. The time-lapse recordings show that growing neurites which encounter a cut end of the DR remain motile, in contrast to the paralysis of neurites which encounter the DREZ. In agreement with our data, two previous *in vivo* studies have also suggested that the adult rat DREZ may provide a "stop signal" for growing axons (Liuzzi, 1990; Liuzzi and Lasek, 1987), analogous to the signals which cause growing axons to halt and form synaptic contacts at appropriate targets. Within the spinal cord, oligodendrocyte cell surfaces are a candidate for this paralysis-inducing activity, since they have been shown to cause long-term paralysis of growing neurites *in vitro* (Bandtlow et al., 1990). However, this is unlikely to be the case at the DREZ, where astrocyte processes abut Schwann cells and these, rather than oligodendrocytes, are therefore the first CNS elements which neurites would encounter as they grow towards the spinal cord along the DR (Fraher and Sheehan, 1987; Berthold and Carlstedt, 1977a).

Although the identity of the DREZ inhibitor(s) remains unknown, we have been able to narrow down the range of likely candidates by making various modifications to the DREZ substrate and/or the culture media in which the applied DRG neurons are grown. Thus, the DREZ barrier is not sensitive to treatment with either Pi-PLC or chondroitinase, suggesting that it is dissimilar to the axon growth inhibitory activities described in the developing chick retina (Stahl et al., 1990), or in the developing mouse neocortex (Emerling and Lander, 1996) and on astroglial scars (McKeon et al., 1995), respectively. In the thalamic and brainstem nuclei of early postnatal mice, fucosylated glycoconjugates are associated with boundary regions which axons do not grow across (Steindler and Cooper, 1987) and also with the PNS-CNS transitional region of the rat trigeminal nerve (Nakagawa et al., 1986). However, we found that the DREZ barrier was unaffected by fucosidase treatment, suggesting that if similar molecules constitute the DREZ barrier, then perhaps their fucose residues

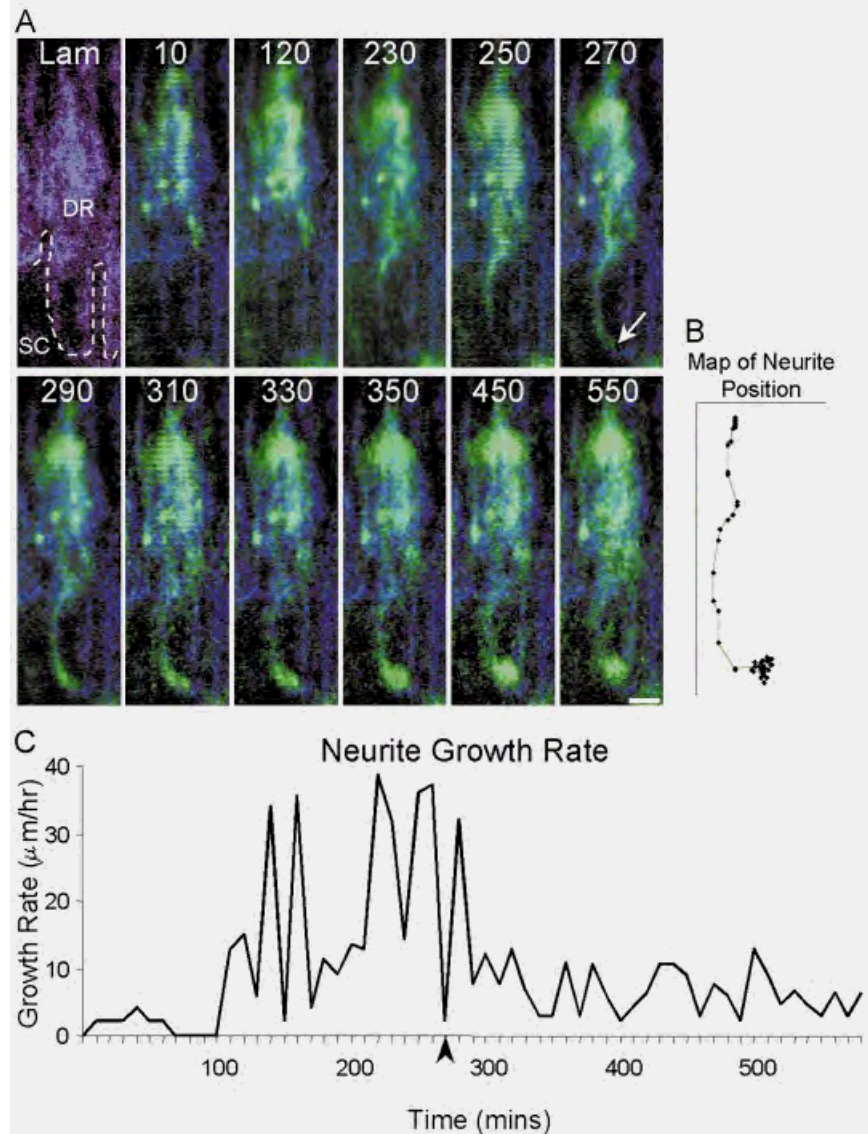


Figure 7.

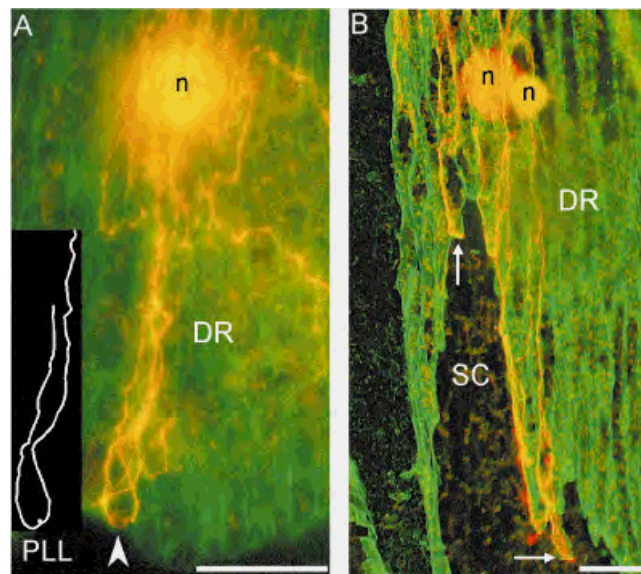


Figure 8.

are not essential for a growth inhibitory function. The DREZ barrier was also unaffected by incubation with suramin or hypertonic saline, suggesting that the inhibitor is unlikely to be bound to the extracellular matrix by heparin-like molecules or via electrostatic bonds, respectively. Finally, the DREZ barrier was unaffected by detergent incubation, suggesting that it is likely to be firmly anchored to the extracellular matrix.

However, both the DREZ barrier and a DRG axon growth-inhibitory activity located on retinal axons, are sensitive to the irreversible proteinase inhibitor PPACK (Baird and Raper, 1995). The idea that a protease activity might be concentrated at the adult rat DREZ is consistent with electron microscopic studies of the cat DREZ (Berthold and Carlstedt, 1977b,c), which suggested that there may be a continual degradation and synthesis of myelin precisely at the PNS-CNS borderline of the DREZ where mitochondria and lipid-like bodies are concentrated. Furthermore, we find that the DREZ barrier is sensitive to organic solvents, such as methanol and/or chloroform, which raises the possibility that the DREZ inhibitory activity could include a detergent-resistant lipid-like molecule, or it becomes denatured by these solvents. Little is known about the possible growth inhibitory influence which lipid-like molecules may have on axonal growth cones, although interestingly it has been shown that the phospholipid, platelet-activating factor (Clark et al., 1995) and the glycerophospholipid, lysophosphatidic acid (Saito, 1997) can both cause growth cone collapse.

By supplementing the culture medium with various drugs and enzymes, we hoped to gain further insight into the receptor mechanisms by which postnatal DRG neuron growth cones recognise the growth inhibitory activity of the DREZ. We used pertussis toxin to disrupt G-protein-coupled signal transduction pathways, since previous studies have shown that pertussis toxin nullifies the axon growth inhibitory activity of GP55, a glycoprotein isolated from chicken brain (Clarke and Moss, 1997), and the NG2 proteoglycan, expressed in a

variety of developing rat tissues (Dou and Levine, 1997). We also supplemented the culture medium with Pi-PLC to remove GPI-linked receptors on growth cones, such as F3/11, which recognises the growth inhibitory ligand tenascin-R (Pesheva et al., 1993; Xiao et al., 1996). Because neither of these treatments promoted neurite growth across the DREZ, G-proteins and GPI-linked receptors are unlikely to be individually essential components of the receptor mechanism for the DREZ barrier.

During DREZ development, PNS and CNS glial processes are separated by a transient population of "boundary cap" cells, which occupy the immature entry sites and are permissive to sensory axon growth (Golding and Cohen, 1997). However, when boundary cap cells disappear (by the end of the first postnatal week in rats) and Schwann cells and astrocytes first come into direct contact, a barrier to growing axons becomes established at the DREZ (Carlstedt, 1988; Golding et al., 1996). If this astrocyte-Schwann cell contact is prevented, then the entry sites remain permissive to axon growth. Thus, experiments in which the formation of the rat DREZ is disrupted by depleting CNS glia in its vicinity with X-irradiation soon after birth, demonstrate that DRG axons lesioned 2–7 weeks after irradiation are able to grow through the astrocyte-depleted entry sites and into the spinal cord (Sims and Gilmore, 1994), well beyond the 1-week postnatal "critical period" for successful regeneration across the DREZ as defined by Carlstedt (1988). Astrocyte-Schwann cell contact might therefore be necessary to trigger the production of axon growth-inhibitory molecules at the DREZ.

However, the persistent expression of an axon growth-inhibitory phenotype at the DREZ into adulthood, in the absence of prior DR injury, suggests that the DREZ barrier may have additional functions, unrelated to constraining axon growth during development. One such function might involve inhibiting the migration of PNS and/or CNS glia within the DREZ. Thus, in the neonatal X-irradiation paradigm described above, if the irradiated DR was not lesioned it was found that Schwann cells would enter and divide within the CNS glia-free regions of the spinal cord (Blakemore and Patterson, 1975; Sims and Gilmore, 1983). Moreover, these intraspinal Schwann cells appeared to halt when they eventually encountered astrocytes at the margins of the irradiated area (Sims and Gilmore, 1983). Similarly, experiments in which Schwann cells and astrocytes are co-cultured, parallel the *in vivo* events of DREZ formation and show that astrocytes compartmentalise Schwann cells, thereby restricting Schwann cell migration (Ghirnikar and Eng, 1995). Those co-culture experiments also revealed an accumulation of CSPG at the sites of astrocyte-Schwann cell contact (Ghirnikar and Eng, 1995), indicating that the two cell types react to each other by producing at least one class of molecule which has previously been demonstrated to act as a barrier to migrating neural crest-derived cells (Kerr and Newgreen, 1997; Pettway et al., 1996; Oakley et al.,

Fig. 7. Neurites stop growing at the adult DREZ. **A:** Time-lapse sequence of CMFDA dye-labelled DRG neurites on a dorsal root (DR) as they grow towards the spinal cord (SC) and encounter the DREZ (dotted line). The first frame shows laminin immunoreactivity (Lam) of the DR, which delineates the DREZ, whilst subsequent frames are a superimposition of laminin immunoreactivity and dye-labelled neurites at various times (shown in minutes). A neurite can be seen to grow along the DR and encounters the DREZ at the point marked with an arrow. Thereafter, the neurite fails to cross the DREZ, retract or turn around. **B:** A map of the position of the growing neurite tip at 10min intervals. **C:** The rate of neurite growth for the sequence in (A) and demonstrates that the neurite grows along the DR in a series of bursts. However, once the neurite encounters the DREZ (at the time point marked with an arrowhead), the growth rate becomes markedly reduced within 20 min. Scale bar = 10 μ m.

Fig. 8. Neurites often turn around at the cut ends of dorsal roots. **A** and **B** show cultured DRG neurons (n) on dorsal root (DR) cryosections, dual-immunostained with anti-laminin (green) and anti-GAP-43 (red) antisera. **A:** Neurites grow along the DR and turn around (arrowhead) when they encounter the interface with the underlying polylysine-coated substrate (PLL) at the cut end of the DR. **Inset** shows a line drawing of the turning neurite for clarity. **B:** By contrast, neurites which encounter the DREZ interface between the DR and the spinal cord (SC) seldom turn around (arrows). Scale bars = 50 μ m.

1994) and growing axons (Grumet et al., 1996; Meiners et al., 1995; Snow et al., 1991). It is therefore tempting to speculate that the primary function of the DREZ inhibitor is to prevent cell migration into or out of the mature spinal cord and that this putative cell migration inhibitor secondarily inhibits axon growth. To test this hypothesis further, it would be of interest to determine whether cryosections of the mature DREZ pose a barrier to the migration of applied Schwann cells or astrocytes *in vitro*.

Within the past few years, it has become clear that the guidance of axons along many developing pathways and within target fields relies upon the interplay between growth-promoting factors and also growth inhibitors and/or repulsive guidance cues (see reviews by Holt and Harris, 1998; Keynes and Cook, 1995). The unique position of the DREZ, forming the borderline between the PNS and CNS, raise the possibility that its axon growth inhibitory activity may be different from those encountered elsewhere within the nervous system. Indeed, our preliminary biochemical characterisation of the DREZ barrier indicate that it is not affected by chemical treatments which are known to neutralise several other axon growth inhibitory activities. Further characterisation of the adult rat DREZ barrier offers the tantalising prospect that appropriate chemical treatment of the DREZ *in vivo* might be able to neutralise the barrier and thus foster the regeneration of DRG axons into the adult mammalian spinal cord. Moreover, our data suggest that such attempts at regeneration would be most likely to succeed if the rhizotomized DREZ were first allowed to undergo reactive changes for several days beforehand.

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