

An Intact Intermediate Filament Network Is Required for Collateral Sprouting of Small Diameter Nerve Fibers

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Expression of the intermediate filament (IF) protein peripherin is initiated during development at the time of axonal extension and increases during regeneration of nerve fibers. To test whether the IF network is essential for neuron process outgrowth in the mature organism *in vivo*, we disrupted endogenous peripherin IF in small-sized dorsal root ganglion (DRG) neurons in transgenic mice via expression of a mutant *peripherin* transgene under control of *peripherin* gene regulatory sequences. Anatomical and functional analyses showed that these neurons send peripheral and central axonal projections to correct targets, express correct neuropeptides, and mediate acute pain responses normally. However, disruption of IF significantly impaired the ability of uninjured small-sized DRG neurons to sprout collateral axons into adjacent denervated skin, indicating a critical role for intact IF in plasticity, specifically in compensatory nociceptive nerve sprouting.

Key words: peripherin; intermediate filament; neurofilament; unmyelinated; collateral sprouting; dorsal root ganglion; primary sensory neuron; pain; nerve growth factor

Introduction

Intermediate filaments (IFs), particularly the neurofilament triplet proteins, are believed to be important in the maintenance of axonal caliber (Julien, 1999). This is of consequence because achievement of sufficient axonal diameter encourages axonal myelination (Voyvodic, 1989), thereby promoting rapid impulse conduction. However, ~65% of primary sensory neurons and most autonomic and enteric neurons possess small caliber axons that are unmyelinated. Nothing is known about the role of IFs in these fibers, although a majority express a distinct neuronal IF protein, peripherin (Leonard et al., 1988; Parysek and Goldman, 1988).

Clues to the function of peripherin IFs may lie in observations that *peripherin* expression correlates with nerve growth. In the developing mouse and rat embryo, *peripherin* expression is initiated in dorsal root ganglion (DRG) neurons because neurites extend to target tissues (Escurat et al., 1990; Gorham et al., 1990; Troy et al., 1990a), and in the adult, *peripherin* expression is

enhanced during regrowth after nerve injury in large-sized neurons of the DRG and in lower motor neurons (Oblinger et al., 1989; Troy et al., 1990b).

A particularly useful paradigm for directly testing the role of peripherin in axonal growth is the skin denervation-induced growth of collateral sprouts from neighboring uninjured neurons (Diamond and Foerster, 1992). In this compensatory sprouting response, which is similar to repair after human skin and nerve damage (Inbal et al., 1987; Ahcan et al., 1998), rodent autonomic and nociceptive functions are restored to damaged skin as a result of collateral sprouting of surviving autonomic (Gloster and Diamond, 1992), small myelinated mechanonociceptive (Nixon et al., 1984), and unmyelinated heat-nociceptive (Doucette and Diamond, 1987) fibers. Both the nociceptive and autonomic sprouting are NGF-dependent processes (Diamond et al., 1987; Diamond et al., 1992b; Gloster and Diamond, 1992). Because *peripherin* expression is known to be NGF-inducible *in vitro* (Leonard et al., 1987) and is particularly prominent in unmyelinated fibers, which mediate pain and temperature sensation, and in the autonomic nervous system, the NGF-driven sprouting paradigm is particularly appropriate for testing the role of peripherin.

To examine the function of peripherin IFs in the growth and function of primary sensory neurons, a mutant *peripherin* transgene under regulatory control of *peripherin* genomic elements was introduced into the germ line of transgenic mice. Studies of the effects of IF disruption as a result of mutant IF subunit gene expression in transgenic mice have substantiated a role for keratin IFs in maintaining the integrity of epithelial tissue (Vassar et al., 1991; Fuchs et al., 1992) and of desmin IFs in maintaining the

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normal structure of muscle (Raats et al., 1996). Dominant-negative studies provide experimental advantages when IF gene knock-outs result, for example, in death of the very cells one wishes to study, as in the case of the *peripherin* knock-out (Lariviere et al., 2002). Additionally, they provided a model for autosomal dominant human diseases caused by mutations in IF genes (Fuchs and Cleveland, 1998; Goudeau et al., 2001). Expression of mutant peripherin devastated the endogenous peripherin network in small-sized DRG neurons. We tested the effects of IF network disruption on functions of these sensory neurons *in vivo* and found that peripherin IFs are required for collateral sprouting.

Materials and Methods

Generation of transgenic mice. All procedures involving mice were approved by the University of Cincinnati Animal Care and Use Committee before the initiation of these studies.

To prepare the Periph/Disrupter (see Fig. 1) transgene, 5.8 Kb of rat *peripherin* gene 5' flanking sequence was fused to a mutant *peripherin* gene at the *Pst*I site at the cap site of the gene (Thompson and Ziff, 1989). The 255 bp deletion in Disrupter removed intron 4 and parts of exons 4 and 5, sequences encoding a portion of the rod domain known to be critical for IF assembly. Fifty-five amino acids, starting at ALDIRA at the beginning of Coil 2 in the rod domain and ending QEMNESR, were deleted. Construction of Periph/Periph-*myc* (see Fig. 1) mice was described by Belecky-Adams et al. (1993). Each transgene contained sequences encoding a human *c-myc* peptide in exon 9 of the *peripherin* gene to aid in immunohistochemical localization of the transgene product.

Transgenic mice were generated according to standard protocols (Chen et al., 1991). Linearized transgene DNA, devoid of plasmid sequences, was injected into the male pronucleus of mouse oocytes obtained from F1 hybrid animals of SJLx57BL6 matings. Mice bearing the transgenes were identified by Southern analyses (Sambrook et al., 1989) of DNA isolated from tails. Founder animals and their offspring were mated to F1 SJLx57BL6 hybrid mice from Jackson Laboratories (Bar Harbor, ME) to maintain a hemizygous line.

Tissue preparation, immunohistochemistry, and antibodies. Tissues used for localization of neuropeptides were derived from mice anesthetized with Avertin (0.125 gm/Kg), perfusion fixed through the left ventricle of the heart with 4.0% paraformaldehyde in 0.05 M phosphate buffer, pH 7.2, postfixed with the same solution for 4 hr, and cryoprotected with 30% sucrose in 0.1 M phosphate buffer for 24–48 hr. Fixed tissues were embedded, frozen in a dry ice–ethanol bath, and stored at -80°C until they were sectioned on a cryostat. Neuropeptide immunoreactivity [Substance P and calcitonin gene-related peptide (CGRP)] was assayed on sections (10 μm) of perfusion-fixed tissue that were incubated in 10% normal goat serum for 20 min and then primary antibody diluted in Tris saline with 0.4% Triton X-100 (TSTX) overnight at 4°C . After washing, tissue was treated with biotinylated IgG (Kirkegaard & Perry, Gaithersburg, MD) at 1:500 in TSTX for 2 hr and Texas Red Avidin at 1:800 in TS for 1 hr. DRG tissue used for all other indirect immunofluorescence was frozen as unfixed material, sectioned by cryostat (6–10 μm), and then fixed in dry -20°C methanol.

DRGs for whole-cell analyses were dissected and dissociated as described by Delree et al. (1989). After trypsin inactivation, DRGs were mechanically dissociated and cytocentrifuged onto glass slides for 6 min at 500 rpm using a Cytospin 2 cytocentrifuge (Shandon-Lipshaw, Pittsburgh, PA). Immediately after cytocentrifugation, cells were fixed in -20°C methanol and then immunolabeled as described by Foley et al. (1991). Whole-gut wall preparations (Bjorklund et al., 1984) were derived from the distal ileum ~ 10 mm proximal to the ileocecal junction and were immunolabeled as described above after fixation in 4.0% paraformaldehyde for 30 min.

Transgene fusion protein was detected using undiluted culture supernatant from a hybridoma secreting a monoclonal antibody to a human *c-myc* peptide (American Type Culture Collection, Manassas, VA) (Evan et al., 1985). An affinity-purified rat peripherin fusion protein antibody made in rabbit was used at a dilution of 1:50 (for sections) or 1:100 (for

cytocentrifuged cells) to detect endogenous and transgene peripherin protein. Culture supernatant containing a monoclonal antibody directed against the 150 and 68 kDa neurofilament proteins (Parysek and Goldman, 1987) was used at a concentration of 3:4 (sections) or 1:2 (cytocentrifuged cells) in TSTX. An antibody raised against a peptide representing the last 19 amino acids of the peripherin protein, which only recognized endogenous peripherin and not transgene protein, was a kind gift from Dr. Lloyd Greene (Columbia University, New York, NY) and was used at a concentration of 1:100. Other antibodies used in this study include: a polyclonal antibody to PGP9.5 (Accurate Chemicals, Westbury, NY), a polyclonal antibody to Substance P (Incstar, Stillwater, MN), and a polyclonal antibody to CGRP (Zeneca, Macclesfield, UK). Some sections were counterstained with Hoechst dye (0.25 $\mu\text{g}/\text{ml}$ of stock; Sigma, St. Louis, MO) at 1:5000 in TS. Photomicrographs of labeled tissue and cells were taken on a Zeiss (Thornwood, NY) Axiophot and recorded on film, and the whole-gut wall preparations were examined in a Molecular Dynamics (Sunnyvale, CA) confocal microscope and recorded digitally.

For data reported in Table 1, IF expression patterns of DRG neurons were assessed by immunofluorescence on cytocentrifuged DRG preparations from three mice of each genotype. For data reported in Table 2, sizes of cells displaying specific IF expression patterns were measured in semithin sections of three mice of each genotype by use of an eyepiece reticle. Neurons were size-classed according to Tennyson and Gershon (1984); small neurons were between 25–400 μm^2 , medium neurons were between 401–900 μm^2 , and large neurons were $>900 \mu\text{m}^2$. The percentage of cells displaying each phenotype was calculated on the basis of the total number of cells counted from all three mice.

Hotplate test. Fifteen disrupter and fifteen wild-type littermate males were selected randomly at 9–11 weeks of age and then individually housed in the testing room at 21°C on a 12 hr light/dark cycle with food and water provided *ad libitum*. All mice were thoroughly conditioned to handling and the hotplate apparatus (Model 39D; Life Sciences, Hialeah, FL) over a 3 week period. The hotplate surface (10.5 \times 11.5 inches) was surrounded by 11 inches high Plexiglas walls to form a chamber. Six days each week, each mouse was held by the same investigator throughout the study for a minimum of 5 min. During the third week, the mice were placed on the hotplate, which had been warmed to 40°C , for 5 min each day. For the fourth and fifth weeks, hotplate data were collected on Monday, Wednesday, and Friday between 4:00 and 10:00 P.M., whereas the standard conditioning continued on remaining days. The surface was maintained at $50 \pm 0.5^{\circ}\text{C}$ (Tjolsen et al., 1991) during testing, which was conducted simultaneously by two investigators (to maximize behavioral observations) who were blind to the genotype of the animals. For the test, each mouse was removed from its home cage, held for 1–2 min until still, and placed on the middle of the hotplate. The mouse was removed from the chamber as soon as the behavioral endpoint of hindpaw lick was seen, or the maximum exposure period of 30 sec had elapsed.

Each mouse underwent four trials per testing day with a 15 min delay between trials. Mice were tested in small groups (of six) so that all data were collected on each animal at the same time each day. To assess the overall performance of each group, the average of each mouse on each day was then averaged with all mice of the same genotype each day. Finally, all 6 d were averaged, and SEM was calculated.

Carmine dye method for measurement of whole-gut transit time. After an overnight fast, allowing only water, and brief anesthesia with methoxy-fluorane, 0.3 ml of a 0.5% solution of carmine red dye was gavaged into the stomach using a 7 French diameter polyvinyl-feeding catheter. The mice continued to fast after introduction of the dye. The whole-gut transit of the dye was measured as the time taken after administration for the colored dye to appear in the fecal pellet. The mice were killed at the end of the study, and the total length of the small intestine and colon were measured on a vertical scale by attaching a known weight to the bottom end and allowing it to hang freely (Helmrich et al., 1996). Transit time was expressed as a function of the total length of intestine traveled (Baldrick et al., 1998).

Collateral sprouting assay. The sprouting assays were performed on age-matched “disrupter mice” and control littermates as described previously (Lourenssen et al., 2000), except that Avertin was used as the surgical anesthesia. Similar numbers of female and male mice were tested

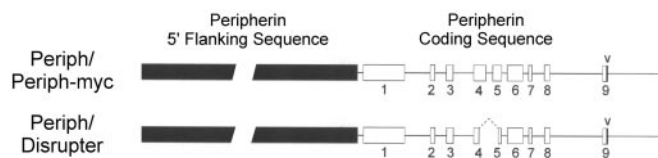


Figure 1. Graphic representation of the transgenes. A dominant-negative mutant *peripherin*/transgene, *Periph/Disrupter*, was constructed by removing intron 4 and parts of exons 4 and 5 (stippled box) from a full-length *myc*-tagged *peripherin* transgene (*Periph/Periph-myc*). To facilitate immunolocalization of transgene protein products, both transgenes contained sequence encoding a human *c-myc* peptide inserted into the 3' end of the coding portion of exon 9 (*v*).

in each group. The field of innervation of the dorsal cutaneous nerve (DCN)-T13 on the mouse back was "isolated" by surgically cutting the DCNs three to four segments above and below T13, effectively denervating the skin surrounding the DCN-T13 nerve field. In this condition, the levels of NGF in the denervated skin rise (Mearow et al., 1993), evoking sprouting of the neighboring nociceptive fibers in the isolated innervation area and causing a progressive expansion of the mechanonociceptive ("pinch") and heat-nociceptive (heat) fields subserved by Aδ and C fibers, respectively (Diamond et al., 1987). The two sensitive areas were mapped separately using the cutaneous trunci muscle reflex, a skin twitch of the underlying cutaneous trunci muscle, evoked by forceps pinch or 60°C probe stimulus (Diamond et al., 1992a). The mapping was performed on days 0 and 21, the field borders were traced and transferred to transparent plastic sheets, and the areas were measured using NIH Image. We evaluated the statistical significance of increases in field sizes at day 21 relative to day 0 in three ways. We analyzed the ratio of expansion by the nonparametric Wilcoxon test and, because the ratios were normally distributed and the groups had equal variances, we also used parametric *t* tests. Additionally, we used ANCOVA analysis of the day 21 area values, with day 0 treated as the covariate to analyze the significance of the raw area data. A sprouting deficit detected in the initial test was confirmed on a second set of mice tested later. Results from both sets of mice were similar and were combined in the final presentation of the sprouting figure.

Results

Generation and initial characterization of mice carrying a mutant *peripherin* transgene

To study the role of the cytoplasmic IF network in the morphology and function of small-diameter primary sensory neurons, a mutant *peripherin* transgene previously shown to disrupt the IF network in PC12 cells (Parysek et al., 1991) was expressed under control of sequences required for cell type-specific expression of the *peripherin* gene (Belecky-Adams et al., 1993) in transgenic mice (Fig. 1). Injection of the transgene into fertilized mouse oocytes resulted in five founder mice, four of which were fertile. Progeny from these four founders expressed the transgene, indicated by *c-myc* epitope expression in nervous tissue as assessed by immunofluorescence.

To assess whether the mutant *peripherin* protein expressed in these mice disrupted the endogenous cytoplasmic filament network, antibodies to *peripherin* and the *c-myc* epitope were used to examine the cytoskeleton in the major *peripherin*-expressing cell type in the adult, the small-sized DRG neuron. In two lines, filamentous labeling in the small-sized DRG neurons was completely absent. Cells from the two fully disrupted lines (disrupter mice) contained globules of *myc*-labeled transgene protein surrounding the nucleus and scattered in the cytoplasm (Fig. 2*A*, arrows). A similar labeling pattern was obtained with a *peripherin* antibody (Fig. 2*B*), indicating endogenous *peripherin* filaments were also fully disrupted. This pattern was in striking contrast to the filamentous labeling pattern seen with either *myc* or

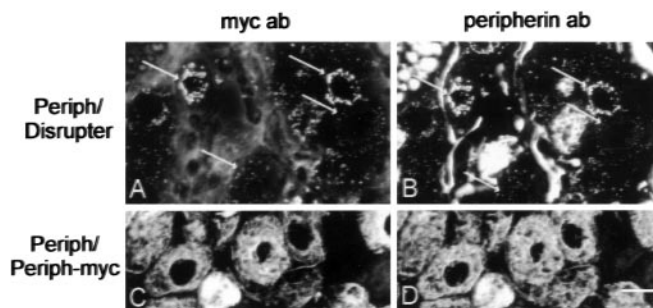


Figure 2. The *peripherin* network is collapsed in the small-sized DRG neurons that express the disrupter transgene. *A–D*, Sections of DRGs derived from adult disrupter mice (*A, B*) and *periph-myc* control mice (*C, D*) double labeled with antiserum to *peripherin* (*B, D*) and antibody to the human *c-myc* tag (*A, C*) showed that small-sized neurons (*A, B*, arrows) contain globules labeled with *peripherin* and *myc* antibodies but no filamentous network. In control DRG (*C, D*), filamentous arrays in small-sized cell bodies are colabeled with both *myc* antibody and *peripherin* antiserum (*C, D*). Scale bar, 10 μ m.

peripherin antibodies in control mice expressing a full-length, nondisrupting, *myc*-tagged rat *peripherin* transgene (Fig. 2*C, D*) (Belecky-Adams et al., 1993). It is likely that all IFs in the small-sized DRG cells were disrupted, because antibody to α -internexin, the only other IF protein known to be expressed in these small-sized cells, also labeled only a globular pattern (data not shown).

Additional analysis of *peripherin* antibody-labeled disrupter mouse DRG sections indicated that, in addition to small-sized DRG neurons (Fig. 3*A*, arrows), globules of *peripherin* were also present in large-sized DRG neurons (Fig. 3*A*, arrowheads). In adult mice, the large-sized cells normally express only the neurofilament triplet proteins [neurofilament (NF)-L, -M, and -H], and this pattern of IF expression was seen in nontransgenic littermate controls (Fig. 3*C, D*) and nondisrupting *peripherin-myc* transgenic controls (Fig. 3*E, F*). Colabeling sections with NF-M and NF-H antibody showed that this ectopic expression of mutant *peripherin* did not disrupt the NF filaments in large-sized DRG neurons of disrupter mice. These experiments showed that, in disrupter mice, filamentous networks were preserved only in DRG neurons that expressed NF-M and -H.

To examine the filament network of disrupter DRG cells with greater resolution, DRGs from adult disrupter mice, nontransgenic littermates, and *peripherin-myc* controls were dissociated, cytocentrifuged onto slides, double-labeled with *peripherin* antiserum and NF-M and -H antibody, and examined by fluorescence and confocal microscopy. These analyses confirmed that the IF network was disrupted in small neurons that expressed only *peripherin* and not NF-M and -H (Fig. 4, top), but was retained in large neurons that coexpressed *peripherin* and NF-M and -H (Fig. 4, bottom).

Neurons with a disrupted IF network innervate target tissues in a manner similar to controls

To determine whether disruption of the IF network altered the differentiation of the small-sized neurons, we assessed the expression of differentiation-specific markers that were important for the function of these neurons. Serial sections derived from disrupter and control L4 and L5 DRGs were analyzed with antisera to substance P or CGRP, and the percentage of neurons that expressed substance P or CGRP was computed by comparison to the total number of neurons determined by staining with Hoechst dye, which stains nuclei. Both control and disrupter DRGs contained substance P in 14% of DRG neurons. CGRP

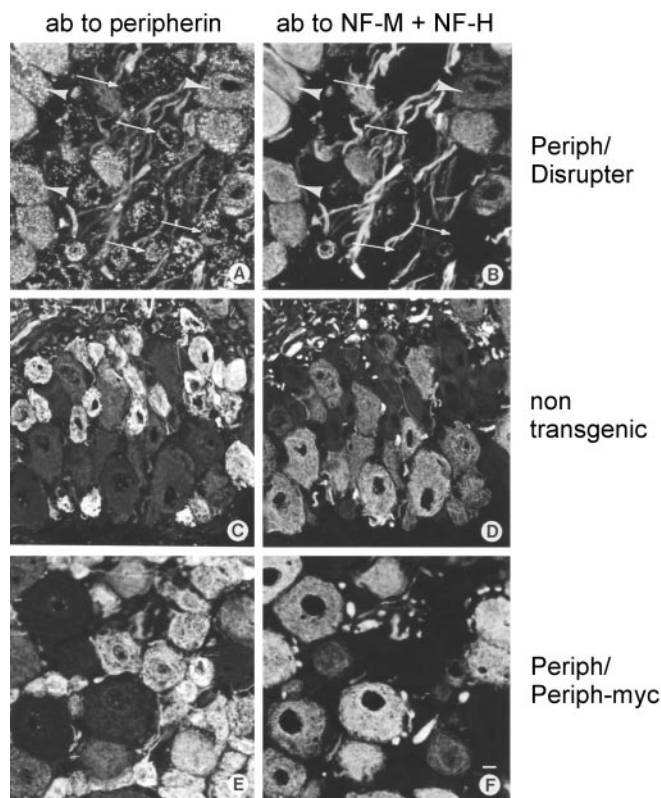


Figure 3. A filament network is detectable in large-sized neurons in disrupter mice. *A–F*, Sections of DRGs derived from disrupter mice (*A, B*), nontransgenic littermates (*C, D*), and periph-*myc* controls (*E, F*) were double labeled with antibodies recognizing peripherin (*A, C, E*) and NF-M and -H (*B, D, F*). Small-sized DRG neurons contain peripherin globules in disrupter mice (*A*) and filamentous networks in control mice (*C, E*) but do not contain an NF-M- or NF-H-containing network (*B*, arrows, *D, E*). However, large-sized neurons in disrupter mice contain not only peripherin-labeled globules but also an IF network that is colabeled with peripherin and NF-M and -H antibodies (*A, B*, arrowheads). Scale bar, 10 μ m.

immunopositive neurons also comprised a similar percentage of DRG neurons in control (34%) and disrupter (37%) mice. As in control mice, neuropeptide immunoreactivity was found primarily in the small-sized DRG neurons in disrupter mice, suggesting that there was no shift in the size class of DRG neuron-expressing substance P or CGRP (Fig. 5*A, B*) in disrupter mice.

To determine whether the small-sized neurons had appropriate central and peripheral terminal projections, sections of the glabrous skin of the footpad and of the lumbar region of the spinal cord were examined qualitatively for the presence of substance P- and CGRP-labeled processes. Antibody to the *myc* tag could not be used for these studies, because the globules of peripherin-*myc* mutant protein were not found in the processes of these cells *in vivo* (data not shown). Sections through the footpad of disrupter mice showed numerous CGRP-positive slender fibers innervating the dermis and protruding into the epidermis. A similar number and distribution of fibers was found in controls (Fig. 5*C, D*). Central innervation patterns of the small-sized neurons were examined in the lumbar region of the spinal cord where CGRP antibody labels only fibers from small-sized DRG neurons that innervate the spinal cord. In control spinal cord, CGRP-immunopositive fibers were found to innervate Rexed's lamina 1 and 2 profusely, with occasional groups of fibers in laminae 3–5 (Fig. 5*F*). In spinal cords of disrupter mice, the spatial distribution and number of CGRP-positive fibers appeared to be similar to control mice (Fig. 5*E*). These results indicate that the percent-

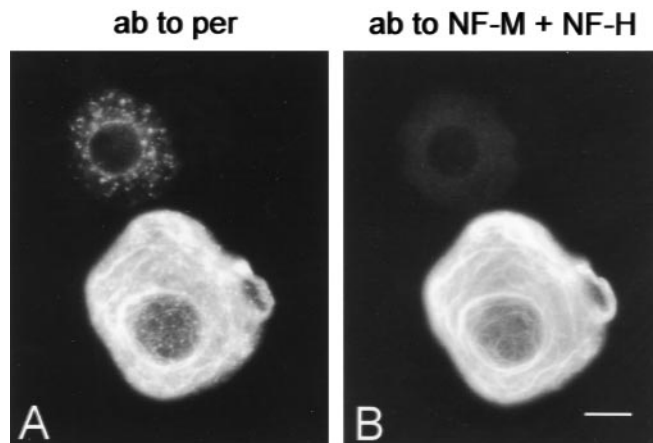


Figure 4. The increased resolution afforded by examining dissociated DRG cells confirms that there is no IF network in small-sized neurons and a peripherin–NF-M-containing network in large-sized neurons. To increase the resolution of the filament network in disrupter DRG neurons, DRGs from disrupter mice were dissociated, cytocentrifuged onto glass slides, and double labeled with antibodies to peripherin (*A*) and NF-M/H (*B*). As observed in sections, no intact peripherin network is seen in small-sized neurons (*A*), and these neurons are not immunopositive for NF-M or NF-H (*B*). However, large-sized neurons still have a filamentous labeling pattern, and this network is colabeled with antibodies recognizing both peripherin (*A*) and NF-M and NF-H (*B*). Scale bar, 10 μ m.

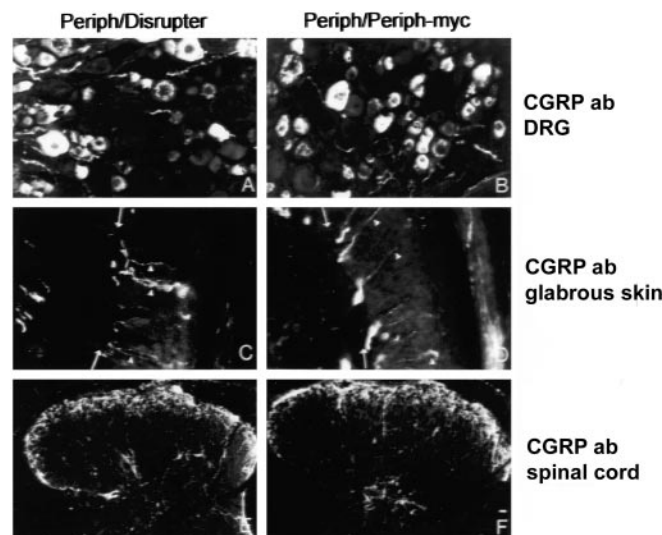


Figure 5. DRG neurons without an intact IF network express correct neuropeptides and show peripheral and central innervation patterns similar to control DRG neurons. *A–F*, Sections of DRG (*A, B*), footpad (*C, D*), and spinal cord (*E, F*) derived from disrupter mice (*A, C, E*) and control littermates (*B, D, F*) were immunolabeled with an antibody that recognizes CGRP. This neuropeptide, normally found in the small-sized neurons of the DRG, shows a similar distribution in disrupter and control DRGs (*A, B*). Qualitatively, similar numbers of peripheral fibers from CGRP-positive DRG cells innervate the dermis of the footpad (*C, D*, arrowheads) (arrows show boundary between epidermis and dermis). Similarly, the central fibers of CGRP-immunopositive cells give rise to an innervation pattern in laminae I and II of the lumbar spinal cord that is similar in both disrupter (*E*) and control (*F*) mice. Scale bar, 10 μ m.

age and size class of neurons expressing the neuropeptides substance P and CGRP were similar in disrupter and control mouse DRG, and that the pattern of innervation of these small-sized neurons appeared to be similar in disrupter and control tissue.

Intact IFs are required for collateral sprouting but not regeneration of small-sized DRG neurons

Although the small-sized sensory neurons appeared to have appropriate anatomical connections in the disrupter mice,

dominant-negative disruption of the IF cytoskeleton requires a high ratio of mutant to wild-type IF protein (Gill et al., 1990), and IF expression in embryonic DRG neurons differs from that in the adult (Goldstein et al., 1996). For these reasons, it is possible that the ratio of mutant peripherin/wild-type IF protein was insufficient to allow for complete disruption of the IF network during the period when neuronal connections were forming. Therefore, it was important to test the role of the IF network in the achievement of neuronal form in adult disrupter mice when the IF network clearly was completely disrupted.

Our initial analysis of neuronal growth focused on a simple regeneration assay. However, preliminary comparison of the number of disrupter and control littermate mice nerve fibers that regenerated 4 mm past a tibial nerve crush site after 56 d (data not shown) indicated no hint of impairment of longitudinal axonal regeneration in disrupter mice. Similarly, there was no gross impairment of the return of skin sensitivity in disrupter mice after nerve crush, although the possibility of some reduction in arborization of regenerated fibers in the skin could not be excluded completely (data not shown). The lack of a significant deficit in regeneration *in vivo*, but robust defect in full neurite tree development *in vitro* (C. A. Ebert, T. L. Belecky-Adams, T. E. Uveges, and C. M. Parysek, unpublished observations), led us to also study neurite growth in a collateral sprouting assay *in vivo*. This assay focuses more precisely than the regeneration assays on unmyelinated C and thinly myelinated Adelta sensory fibers (Nixon et al., 1984; Doucette and Diamond, 1987), the major fiber types associated with the sizes of *peripherin*-expressing neurons (Ferri et al., 1990) that had disrupted IFs. [The large myelinated mechanosensory touch fibers do not undergo spontaneous sprouting in adult rats (Jackson and Diamond, 1984)]. It was plausible that peripherin had a role in sprouting because collateral sprouting is an NGF-dependent process (Diamond et al., 1987), and the *peripherin* gene has been shown to be regulated by NGF *in vitro* (Thompson et al., 1992). Therefore, the capacity of the unmyelinated heat-nociceptive fibers and thinly myelinated pinch-sensitive fibers to sprout into denervated skin was tested and compared in disrupter and control mice.

The field of skin on the back of the mouse that was responsive to either pinch or heat stimuli and served by an intact cutaneous branch of the T13 nerve was tested on day 0, the day in which cutaneous branches immediately above and below T13 were severed, and on day 21 after surgery, and the degree of expansion of the sensitive nerve field compared between age- and sex-matched control and disrupter mice was measured. The average day 0 field sizes were slightly larger in disrupter mice (mean, 0.58 cm² for heat, 1.095 cm² for pinch) compared with control mice (mean = 0.46 cm² for heat, 0.943 cm² for pinch) before sprouting. Significantly, however, the areas of the T13 heat-sensitive fields expanded by approximately twofold (average ratio of expansion for day 21/d 0, 1.87) in control mice ($n = 18$) but only $\sim 1.34\times$ in disrupter mice ($n = 18$; $p = 0.0008$; parametric t test for ratio). The expansion of the pinch-sensitive fields was also impaired significantly in disrupter mice ($1.49\times$) compared with control mice ($1.81\times$; $p = 0.013$; parametric t test for ratio) (Fig. 6). The ratio data were significant by both the parametric t test for ratio and the nonparametric Wilcoxon test for ratio. Additionally, analysis of the raw area data by ANCOVA, with the day 0 value treated as covariate, showed significant differences ($p = 0.0013$) in the heat but not pinch areas. These data strongly suggest that peripherin IFs are required for the sprouting of new branches from uninjured, nociceptive peripheral nerve fibers.

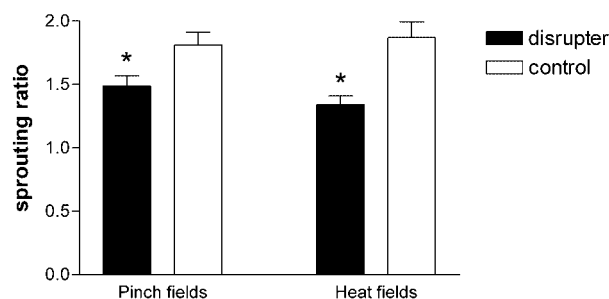


Figure 6. Collateral sprouting of heat- and pinch-sensitive, small-sized primary sensory neurons in disrupter mice is significantly reduced relative to the same population of neurons from mice with wild-type IFs. After denervation surgery that creates an “island” of normally innervated skin in a region of nonsensitive skin, sprouting of nociceptive fibers causes the expansion of initially measured (day 0) heat-sensitive and pinch-sensitive areas from the island. Sensitive areas were again measured at 21 d and compared with the size of the original areas. The average degree of expansion of heat sensitivity in control mice was $1.87\times$ but was only $1.34\times$ in age-matched disrupter mouse littermates ($p = 0.0008$). Expansion of the pinch-sensitive fields was also reduced significantly in disrupter mice ($p = 0.017$). The columns show mean \pm SEM ($n = 18$).

Intact IFs are not required for other functions of peripherin-expressing neurons

To assess the generality of the requirement for intact IFs in neuronal function, we examined additional physiological functions of *peripherin*-expressing neurons. Small-sized DRG neurons are responsible for transmitting information concerning painful stimuli from the periphery to the CNS. We tested whether mice bearing the disrupter transgene were as responsive to pain stimuli as their normal littermates by comparing the latency with which disrupter and control mice respond to being placed on a 50°C hotplate. The mean latencies of disrupter and littermate control mice for response to the hotplate were similar (18.3 sec; SEM, ± 0.56 sec; $n = 15$) versus (18.7 sec; SEM, ± 0.5 sec; $n = 15$), indicating that IF disruption does not interfere with response to a 50°C heat stimulus.

We also determined whether there were abnormalities in enteric neurons, the other major neuron type that expresses peripherin as a major IF subunit. Confocal analyses of whole-gut wall preparations from disrupter mice showed that, like in sensory neurons, mutant peripherin was concentrated in neuronal cell bodies, and the IF network in *peripherin*-expressing gut neurons was disrupted (data not shown). Colabeling of such preparations with anti-PGP9.5, which labels all neurons (Wilkinson et al., 1989), showed that neurons expressing mutant *peripherin* have processes, and that the overall structure of the gut nervous system appeared indistinguishable between disrupter and control mice (data not shown). To determine whether the disrupted IF cytoskeleton in gut neurons affected their function, we compared the gut transit time relative to intestinal length of disrupter and wild-type littermate mice. The movement of carmine dye, gavaged into the stomach, was measured by determining the time at which it first appeared in fecal pellets. The gut transit rate averaged 0.73 cm/min in disrupter mice (range, 0.33–0.97 cm/min; $n = 8$) and 0.70 cm/min in wild-type littermates (range, 0.42–0.96 cm/min; $n = 9$), and there were no significant differences in the length of the intestinal tract (small intestine plus colon) between the two groups of mice.

The alteration in large-sized neuron phenotype in disrupter mice

Expression of *peripherin* in large-sized DRG neurons of disrupter mice was unexpected. We confirmed that both mutant and en-

Table 1. Percentages of cells expressing peripherin, NF-M, or both in the total DRG neuron population from disrupter and control mice

| Mouse genotype | Peripherin + | NF-M + | Coexpression of peripherin and NF-M | Total number of neurons counted |
|-------------------------------|--------------|--------|-------------------------------------|---------------------------------------|
| Periph/disrupter transgenic | 56.6% | 4.6% | 38.9% | <i>n</i> = 19,727 neurons from 3 mice |
| Periph/ <i>myc</i> transgenic | 68.9% | 24.7% | 6.4% | <i>n</i> = 21,400 neurons from 3 mice |
| Nontransgenic littermate | 66.5% | 21.9% | 11.6% | <i>n</i> = 16,969 neurons from 3 mice |

Table 2. Percentages of large-sized DRG neurons expressing only NF-M or both peripherin and NF-M in disrupter and control mice

| Mouse genotype | NF-M + | Coexpression of peripherin and NF-M | Number of neurons measured |
|-------------------------------|--------|-------------------------------------|----------------------------|
| Periph/disrupter transgenic | 1.0% | 33.3% | 983 |
| Periph/ <i>myc</i> transgenic | 16.5% | 9.0% | 1030 |
| Nontransgenic littermate | 18.3% | 5.0% | 1021 |

ogenous peripherin were included in the peripherin antibody-labeled globules by labeling DRG sections from disrupter mice with an antibody to the carboxyl terminal 19 amino acids of peripherin, which were present only in endogenous peripherin (data not shown). Examination of DRG neuron preparations revealed that coexpression of *peripherin* and *NF-M* was increased (Table 1) in the large-sized neurons (900 μm^2 or greater) (Table 2) of disrupter mice as compared with wild-type littermates and peripherin-*myc* controls. This inappropriate expression could reflect alterations in the development of the large-sized cells or represent a normal injury response (Wong and Oblinger, 1990) to an altered environment of these cells in disrupter mice.

Discussion

To define the role of IFs in neurons with small-caliber projections, we tested the effect of disrupting these filaments through targeted expression of a mutant *peripherin* transgene. Expression of mutant peripherin induced no detectable morphological defect in *peripherin*-expressing enteric neurons or in the projections of small-sized DRG neurons to the skin or spinal cord in the adult mouse. In addition, disruption of IFs in small-sized DRG neurons did not interfere with their ability to mediate a response to a 50°C heat stimulus. However, analysis of IFs in cultured embryonic DRG neurons from disrupter mice suggested that IFs may not have been disrupted completely in these neurons during the period when axonal projections formed and thus did not provide an adequate test of the importance of IFs in the development of processes of these cells. Therefore, to determine the effect of IF disruption on nerve growth, we analyzed the ability of adult DRG sensory neurons in which IFs were disrupted fully to undergo either regeneration after nerve crush or peripheral collateral sprouting from noncrushed axons in response to adjacent skin denervation. Strikingly, IF disruption profoundly reduced the ability of these axons to sprout collateral branches.

Collateral sprouting from uninjured neurons is distinct from axonal regeneration, which is triggered by axonal damage, and from the type of sprouting ("regenerative sprouting") that is confined to the growing tip of a regenerating axon (Diamond et al., 1992c). The collateral sprouting assay in rodents specifically tests the capacity of undamaged myelinated and unmyelinated nociceptive nerve fibers in the skin to expand their terminal trees of free nerve endings in response to denervation of adjacent skin, a process that is absolutely dependent on NGF (Diamond et al., 1987, 1992a; Gloster and Diamond, 1992). Cutaneous NGF levels increase after denervation in part because of elimination of NGF uptake into nerves (Korsching and Thoenen, 1985), and in part

because its production, largely in basal epithelial cells, increases after denervation (Mearow et al., 1993). NGF acts as a trophic agent that drives the sprouting of undamaged nociceptive neurons but not axonal regeneration triggered by nerve damage (Diamond et al., 1992a, b, c).

The collateral sprouting assay is particularly useful for testing neuronal function in disrupter mice, because mutant peripherin disrupted IFs precisely in those cells with the greatest capacity for collateral sprouting and for which the expansion of heat sensitivity is a precise measure of sprouting. In wild-type mice, the only neurons in the DRG that subserve heat nociception are the *peripherin*-expressing small-sized primary sensory neurons of the DRG (Ferri et al., 1990), 80% of which are heat nociceptors (Kirschstein et al., 1999). In sectioned DRG and cytocentrifuged single-cell preparations, all peripherin in small cells appeared as dots distributed throughout the cell body (Figs. 2, 3, 4). α -internexin, the only other IF subunit known to be expressed in some of these cells (Molliver et al., 1995), was not filamentous; it was only found colocalized in dot-like structures with peripherin (data not shown). We tested for spurious expression of other IF genes including vimentin, glial fibrillary acidic protein, and NF-L in these cells and found none (T.B.-A. and L.M.P., unpublished observations). The lack of peripherin in axons was severe because sections of the peripheral nerve of disrupter mice were devoid of peripherin (data not shown). Indeed, to visualize the projections of these neurons, it was necessary to use Substance P and CGRP as markers. Thus, in the adult mouse, IF protein was restricted to dots in the cell body of small-sized DRG neurons and was not detectable in axons or terminal projections of these neurons. Collateral sprouting of this heat-sensitive cell type in disrupter mice was severely inhibited, as indicated by every statistical method used to analyze the data, strongly implicating a role for IFs in this process.

A second type of neuron that sprouts collaterals, the intermediate-size class of DRG neurons with finely myelinated axons that primarily subserves mechanonociception, or pinch (Nixon et al., 1984), also expresses mutant peripherin in disrupter mice. Collateral sprouting of these pinch-sensitive fibers was reduced significantly in disrupter mice, but the fact that one of the statistical tests, the analysis of the original pinch-sensitive areas by ANCOVA, did not rise to the level of significance suggests that the effects of IF disruption on the pinch nociceptors was not as robust as the effects on heat nociceptors. The difference in impairment between the thermal nociceptors and the mechanonociceptors may be because intermediate-sized, but not small, DRG neurons coexpress NF-L, -M, and -H proteins (Ferri et al., 1990), which may have the ability to rescue the IF disrupting effects of mutant peripherin expression in these cells (Fig. 4). Thus, IFs may not have been disrupted in some (the larger) of the fibers tested in the pinch assay.

The relationship of IF disruption to the sprouting impairment is specific and strong. The number of small-sized neurons that furnished CGRP- and Substance P-positive free nerve endings to the skin was normal in disrupter mice. These cells supported a response to an acute heat stimulus that was in the normal range.

In addition, whole-gut motility, controlled in part by *peripherin*-expressing enteric neurons, was unaffected. Therefore, all of these functions either do not depend on axonal IF or are rescued by nonfilamentous IF protein in the cell body, or perhaps more likely are significantly less-sensitive indicators of disrupted IFs than sprouting. However, the specific defect in collateral sprouting provides a specific mechanism for testing the role of *peripherin* IFs in cell physiology, a step forward in analysis of IF function.

IFs were required for expansion of peripheral neurite trees from uninjured small-sized DRG neurons *in vivo* (this study) and for full neurite tree development *in vitro* (Ebert et al., 2003). A previous study specifically linked the loss of NF-L, which severely limits the number of IFs in axons (Zhu et al., 1997), with diminished neurite tree development in large motor neurons in NF-L knock-out mice (Zhang et al., 2002). *In vitro*, IF loss was specifically linked to the reduction of axonal growth in *Xenopus* neurons (Walker et al., 2001) and process growth in astrocytes (Lepekhn et al., 2001). Involvement of IF proteins in the development of neurite trees is also supported by data showing that a particular phosphorylated epitope of NF-M is present only in turning or branching neurites *in vitro* (Landmesser and Swain, 1992). In disrupter mice, the defect in collateral sprouting of nociceptive axons suggests a specific role for IF networks in sprouting type growth of undamaged fibers that is NGF-dependent. This suggestion takes account of the finding that NGF-independent growth (Diamond et al., 1987, 1992b), as tested in the regeneration assays, was unimpaired in disrupter mice.

Peripherin could be required for transduction of the NGF signal or for the axonal responses that are triggered by NGF. The possibility that peripherin is important for some aspect of NGF signaling is supported by known physical interaction of peripherin with the intracellular domain of tyrosine receptor kinase A (*trkA*) (MacDonald et al., 1999). Peripherin could serve as a scaffolding protein in the receptor complex, similar to the proposed role of keratin 18 in the death receptor complex (Inada et al., 2001), or could be important for uptake or movement of *trkA* vesicles essential for some forms of *trkA* signaling (Ginty and Segal, 2002). The intracellular domain of *trkA* is on the outside of the vesicle and available for interaction with motor molecules such as dynein (Yano et al., 2001; Bhattacharyya et al., 2002) and molecular components that might facilitate their retrograde transport via dynein, like peripherin. A highly similar protein to peripherin, vimentin, is already suspected to interact with dynein (Helfand et al., 2002). Alternatively, the role of peripherin may be in the effector side of the NGF-signaling pathway. Although it is less easy to visualize how cytoskeletal requirements may differ for collateral sprouting type growth versus regeneration of a main axon [however, there is evidence (Smith and Skene, 1997) for a differential expression of growth-related genes in the two growth states], it is reasonable to consider that the configuration of slender collaterals at sometimes right angles to the main axon may require IFs for flexible support struts or aid in physically directing needed materials, such as membrane components or mitochondria, into sprouts. The identification of a role for IFs in collateral sprout formation makes it possible to directly test these hypotheses for IF function in these fibers.

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