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Dorsal root ganglion neurons require functional neurotrophin receptors for survival during development

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SUMMARY

Neurotrophins are the most profound known regulators of survival in the developing peripheral nervous system. Within dorsal root ganglia, the signalling receptors for the different members of the neurotrophin family are distributed in distinct patterns suggesting regulation of different functional classes of sensory neurons. Abnormalities observed in neurotrophin receptor mutant mice have confirmed this idea. Both *trk*A (−/−) and *trk*C (−/−) mice have striking neurological defecits referrable to subpopulations of DRG neurons which have distinct axon projections in the periphery. These results thus generalize concepts of dependence on target-derived factors based on extensive work with the prototypical neurotrophin, nerve growth factor. Further analysis of these animals also provides evidence for more complex developmental mechanisms including dependence on locally synthesized neurotrophins at early developmental stages and plasticity of neurotrophin receptor expression.

1. DEPENDENCE OF NEURONS ON TARGET-DERIVED FACTORS DURING DEVELOPMENT

The dependence of peripheral neurons on tissues that they innervate, established on the basis of target ablation studies carried out in chick embryos during the 1930s and 1940s, is now considered to be a fundamental principle of neural development. A molecular basis of this interaction for certain classes of neurons was elucidated in the 1950s when the molecule nerve growth factor (NGF) was discovered and subsequent experiments demonstrated that NGF mediated survival and growth-promoting effects for sympathetic ganglion neurons and certain populations of sensory neurons in the dorsal root and trigeminal ganglia (for a review see Levi-Montalcini 1987). The generality of the phenomenon of neuronal death after target deprivation led to the widely held view that survival-dependence of neurons on target-derived molecules during development is a general property of neurons and that additional molecules would be identified which mediated this function for non-NGF dependent classes of neurons. The localization of these survival functions to innervated tissue presumably allows some matching of populations of cells that proliferate independently, but whose approximate numbers must be matched in some way (Barde 1994).

In a landmark study published in 1982, a new factor, brain-derived neurotrophic factor (BDNF), purified from brain on the basis of its ability to promote survival of sensory neurons was described (Barde *et al.* 1982). Cloning of the gene encoding this factor rapidly lead to the discovery of an NGF family of molecules, the neurotrophins (Liebrock *et al.* 1989; and for a review see Barde 1992). It was immediately hypothesised that these molecules might regulate survival of the non-NGF dependent classes of neurons, particularly in the peripheral nervous system. In support of this view, *in vitro* studies employing explants or dissociated neurons from peripheral ganglia demonstrated that the different neurotrophins show striking specificty for particular ganglia in their ability to promote survival and neurite outgrowth (for a review see Davies 1992). The identification of the signalling receptors for the neurotrophins as the Trk family of receptor tyrosine kinases has further supported this view (for a review see Barbacid 1994). Genes coding for these receptors are expressed in distinct patterns, particularly in the peripheral nervous system, consistent with the idea that BDNF, NT-3 and NT-4 regulate the survival of non-NGF dependent neurons (Ernfors *et al.* 1992; and for a review see Snider 1994).

The dorsal root ganglion system has proven to be an outstanding model system for delineating functions of the different members of the neurotrophin family. Each DRG is comprised of more than 20 functional classes of neurons, each with a distinct pattern of peripheral and central projections (Perl 1992; Willis & Coggeshall 1992). Work *in vitro* has strongly suggested that different populations of DRG and trigeminal neurons, indeed, have distinct neurotrophin require-

ments during development (for a review see Davies 1992). The generation of neurotrophin and Trk null mutant mice will now allow a precise determination of the neurotrophin requirements of the major classes of DRG neurons *in vivo*.

2. EXPRESSION OF NEUROTROPHIN RECEPTORS BY DRG NEURONS

Expression patterns of neurotrophin receptor mRNA and protein in rat and mouse DRGs strongly support the idea that neurons of different functional class are regulated by different members of the neurotrophin family. A rough scheme of the pattern of Trk mRNA and protein expression in association with several well charcterized DRG neuronal cytochemical markers is shown in figure 1. Size-frequency histograms of soma cross-sectional areas of neurons expressing the high affinity NGF receptor TrkA show that these are predominately medium-sized and small DRG neurons (Mu *et al.* 1993; McMahon *et al.* 1994; see also Molliver *et al.* 1995). Studies at both the mRNA and protein level suggest that TrkA-expressing neurons in adult rat and mouse DRG represent 40–45% of the neurons with little difference in percentages between thoracic and lumbar levels. (Verge *et al.* 1992; Averill *et al.* 1995; Molliver & Snider 1995; Molliver *et al.* 1995).

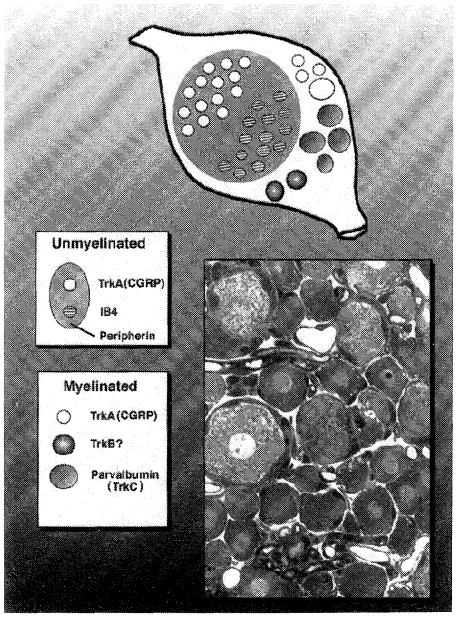

Figure 1. Trk expression in adult rat DRG. Schematic of DRG organization in terms of neurotrophin receptor expression. This scheme is for adult rat thoracic DRG. Inset shows large light (myelinated) and small dark (unmyelinated) neurons that have formed the traditional morphological classification of DRG neurons. Large light cells express the heavy chain of neurofilament in their cytoplasm whereas the small dark neurons express the intermediate filament protein, peripherin. There appear to be four major classes of DRG neurons. Neurons expressing TrkA mRNA and protein represent approximately 45% of DRG neurons. TrkA neurons have small and medium size soma cross-sectional areas. Most of these are unmyelinated, although a few myelinated (NFH expressing) DRG neurons are also labeled. The TrkA population is almost identical to the population of DRG neurons which express CGRP. Note that TrkA does not label the entire unmyelinated population. Non-TrkA unmyelinated neurons probably do not express any neurotrophin receptor and are labelled by the lectin BSI-B4. Neurons expressing TrkC represent 18% of adult rat DRG neurons and have large and medium-sized soma areas. A subset of this population is labeled by the calcium binding protein parvalbumin. Neurons expressing TrkB in isolation represent approximately 5% of adult DRG neurons and are predominantly of intermediate size soma areas. Chemical markers for the TrkB expressing population and smaller TrkC neurons have not yet been identified.

TrkC-expressing neurons within the DRG represent a numerically smaller population with a different soma size profile. These neurons have predominately medium and large cell soma areas and include the largest neurons in the DRG (Mu *et al.* 1993; McMahon *et al.* 1994). Interestingly, there is some degree of co-localization between TrkA and TrkC. Neurons expressing mRNA for both receptors make up roughly 5% of the TrkA population, but almost 15% of the TrkC population (Wright & Snider *et al.* 1995). The functional significance of this dual expression is unknown. Patterns for the TrkC isoforms containing and lacking tyrosine kinase domains differ little in DRG (McMahon *et al.* 1994).

A third population of DRG neurons expresses mRNA for the catalytic isoform of TrkB at high levels. This is a population with predominantly intermediate cell sizes and constitutes less than 10% of neurons in the ganglion (Mu *et al.* 1993; Wright & Snider 1995). These neurons do not express any other Trk at appreciable levels (Wright & Snider 1995). It is now clear that, in addition to these high-level TrkB expressors, as many as 25% of DRG neurons express mRNA for the catalytic isoform of TrkB at low levels (McMahon *et al.* 1994). These are co-localized with both TrkA population, particularly in visceral afferents and with the TrkC population in muscle afferents. The non-catalytic isoform of TrkB is expressed extensively by non-neuronal cells within the ganglion (Carroll *et al.* 1992; Armanini *et al.* 1995).

Finally, a fourth broad population comprising between 25% (Wright & Snider 1995) to 35% (McMahon *et al.* 1994) of DRG neurons expresses no member of the neurotrophin receptor family in adulthood. These neurons are of exclusively small diameter (McMahon *et al.* 1994; Wright & Snider 1995). As expected, the low-affinity neurotrophin receptor p75 is expressed by most of the Trk-expressing cells, and by none of the non-Trk-expressing population. However, there exists a population of roughly 50% of TrkC-expressing neurons which do not express p75 (Wright & Snider 1995). Whether this group represents a distinct functional class is unknown. Overall, expression patterns in other peripheral ganglia conform to the general pattern outlined above and support the idea that different functional classes of peripheral neurons will have different profiles of neurotrophin requirements (for a review see Snider 1994).

Studies at the protein level have confirmed this broad demarcation of different cell subclasses and have provided additional information about the phenotype

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of neurons expressing particular neurotrophin receptors. Most of the work to date has been reported with antibodies generated against TrkA (Clary *et al.* 1994; Averill *et al.* 1995; Molliver *et al.* 1995). TrkA+ cells have distinct cytochemical characteristics. First, only a small subset of TrkA neurons express neurofilament heavy chain (NFH) which is thought to be a marker for myelinated DRG neurons (Lawson 1992; Averill *et al.* 1995; Molliver *et al.* 1995). The subset of neurons in which TrkA and NFH are co-localized presumably make up the nociceptive Ad population. The majority of TrkA-expressing cells are NFH negative suggesting that they are unmyelinated.

As expected, on the basis of high affinity binding and peptide co-localization studies (Verge *et al.* 1989), virtually all neurons which express CGRP and substance P are TrkA positive (Averill *et al.* 1995). Consistent with this idea is the demonstration that these two molecules have very similar patterns of expression in lamina I and the outer aspect of lamina II in the spinal cord (Molliver *et al.* 1995). However, a large population of DRG neurons with small soma areas exists which do not express TrkA mRNA or protein (McMahon *et al.* 1994; Averill *et al.* 1995; Molliver *et al.* 1995; Silos-Santiago *et al.* 1995). Most, if not all, of these non-TrkA-expressing, small DRG neurons bind BSI-B4, a specific isolectin from *Bandiera Simplicifolia*.. It appears that the population of neurons binding the isolectin BS1-B4 is identical to the population of neurons in the ganglion that do not express any member of the Trk family or p75 (McMahon *et al.* 1994; Wright & Snider 1995; Silos-Santiago *et al.* 1995). Interestingly, BSI-B4 binds intensely to the inner part of lamina II, a termination region different from CGRP and TrkA-expressing neurons, which project to lamina I and IIo.

Developmental patterns of neurotrophin receptor expression are fully consistent with the idea that they mediate survival functions at appropriate times during what is broadly considered to be periods of naturally occurring cell death. Thus, in rat dorsal root ganglia all three Trks and p75 are expressed in DRGs as early as embryonic day 11, a stage when ganglion cell precursors are still proliferating (Zhang *et al.* 1994; and see also White *et al.* 1995). There is evidence, in mouse, that TrkC is expressed even earlier, as neural crest neurons are migrating to form the ganglion (Tessarrollo *et al.* 1993). It is not possible to say whether the patterns of expression are distinct at the early developmental stages. A full description of patterns of ligand expression is beyond the scope of this paper, but all neurotrophins are expressed at early developmental stages in distinct patterns in proximity to the peripheral projections of DRG neurons (for representative descriptions see Schectersen & Bothwell 1992; Henderson *et al.* 1993). Also of note is that both BDNF (Ernfors & Persson 1989; Schecterson & Bothwell 1992) and NT-3 (Elkabes *et al.* 1995) are expressed within DRGs during development and BDNF is expressed by Schwann cells (Acheson *et al.* 1991). These findings suggest that DRG neurons may acquire neurotrophins not only from target fields but also via paracrine and even autocrine mechanisms.

3. NEUROTROPHIN RECEPTOR GENE TARGETING ELIMINATES SPECIFIC POPULATIONS OF DRG NEURONS

Immune deprivation of NGF *in utero* achieved in the early 1980s established that the majority of DRG neurons require NGF for survival during development (Johnson *et al.* 1980; and for a review of the primary literature see Ruit *et al.* 1992). Neurons with small soma and axon diameters and those expressing substance P appeared to be most vulnerable. Furthermore, animals borne to autoimmune mothers exhibited behavioural insensitivity to painful stimuli, suggesting that DRG neurons mediating nociception require NGF. It was unclear from the original studies whether remaining DRG neurons did not require NGF for survival during development, or were somehow not susceptible in the experimental paradigms used, perhaps because they pass through a phase of NGF dependence at an earlier developmental stage. In 1992, a study reporting a paradigm of *in utero* NGF deprivation in rats using an antibody injected as early as E15 was reported (Ruit *et al.* 1992). It was demonstrated that NGF-dependent neurons were primarily small diameter neurons which projected to lamina I and II of the dorsal horn. DRG neurons projecting to other regions of the spinal cord were not obviously affected by NGF deprivation. It was hypothesised that remaining neurons in the DRG would express receptors for, and be dependent upon, more recently identified members of the neurotrophin family.

There was difficulty, initially, in generating antibodies to BDNF and NT-3. Thus similar types of antibody deprivation studies for these molecules were not carried out until recently (Gaese *et al.* 1994; Oakley *et al.* 1995) The perfection of gene targeting techniques, however, has lead to definitive information about the classes of primary sensory (as well as other peripheral ganglion neurons) that are dependent on different members of the neurotrophin family. Mice with mutations in the known neurotrophin and neurotrophin receptor genes have been generated, allowing a relatively complete description of the dependence of DRG neurons on neurotrophin/Trk signalling. We have collaborated with Dr Mariano Barbacid in characterizing animals in which Trk genes have been targeted, and consequences of these mutations for survival of different classes of DRG neurons will be described below.

(*a*) *TrkA null mutants*

Work with *trk*A (−/−) mutants has largely confirmed the previous work with direct injection of NGF antibodies or rendering pregnant mothers autoimmune NGF. DRG neuron loss in these animals is massive (Smeyne *et al.* 1994; Silos-Santiago *et al.* 1995). Neuron loss in the L4 and L5 lumbar ganglia is 82%. Interestingly there is little loss in heterozygote animals. Size-frequency histograms show that all of the smallest neurons as well as some medium-sized neurons are lost in these animals (figure 2). Similarly, electron micrographs of thin sections of dorsal roots have shown that

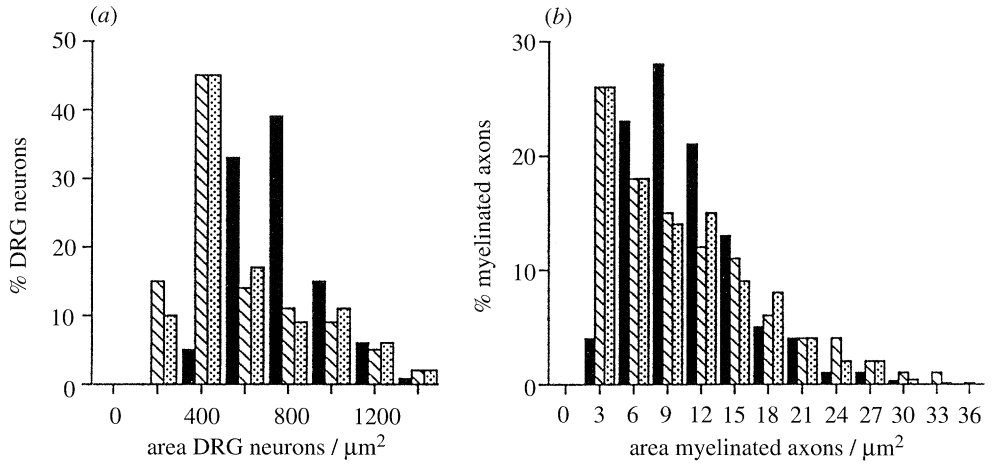

Figure 2. Size-frequency histograms of PN14 L4-L5 DRG neurons and axons in trkA (+/+), trkA (+/−) and trkA (−/−) mice. Shading indicates trkA −/−; stripes indicate trkA +/+; stippling indicates trkA +/−.

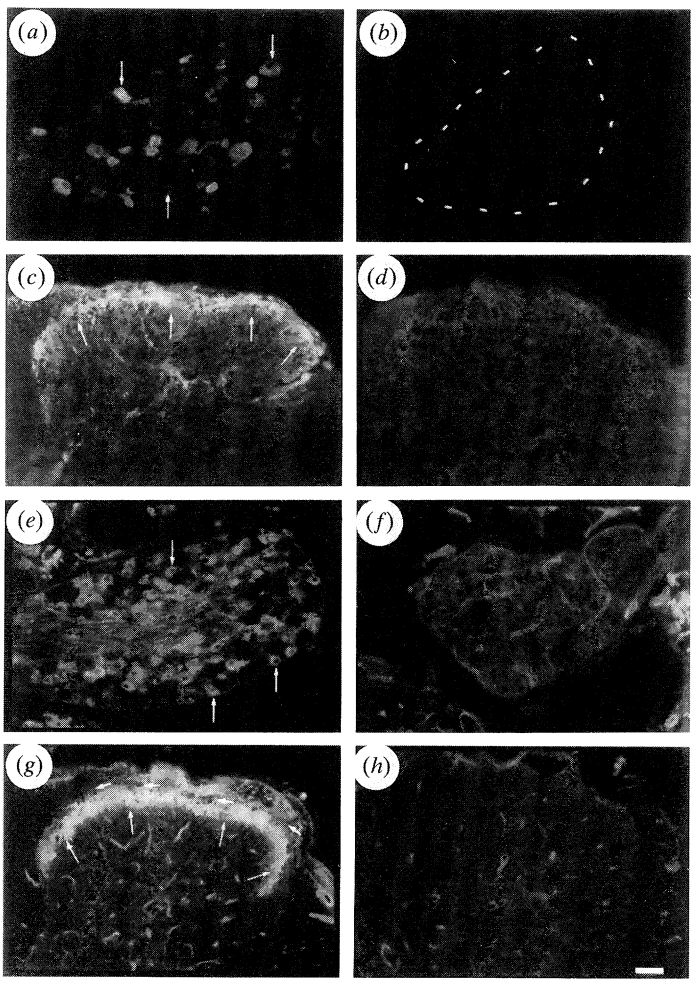

Figure 3. Both TrkA and non-TrkA expressing small DRG neurons are lost in TrkA −/− mice. Transverse sections of DRGs and spinal cord in wild-type (left) and TrkA (−/−) mutant animals (right) illustrating immunoreactivity for CGRP and BS1. (*a*) DRGs from wild-type mice showing CGRP-IR neurons (arrows). (*b*) In trkA −/− mutants all CGRP positive DRG neurons have been eliminated. The border of the DRG is shown in dashed lines. (*c*) CGRP immunoreactivity (arrows) is present in the superficial laminae, I and II_o of the dorsal horn as well as in lamina 5. CGRP is completely absent from the spinal cord of the −/− mutant. (*e*) BS1 labels a population of small DRG neurons in wild-type mice. (*f*) All BS1 positive DRG neurons are absent in trkA −/− mice. (*g*) BS1 labels the entirety of lamina II of the superficial dorsal horn (lower arrows). Lamina 1 is not stained (small upper arrows). (*h*) BS1 labelling in spinal cord has also been eliminated in trkA −/− mutant mice. Scale bars 50 μM.

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virtually all unmyelinated and approximately 50% of myelinated axons are lost (Silos-Santiago *et al.* 1995). The lost myelinated axons were almost exclusively in the 2–5 μm (Ad) range and roughly 75% of axons in that size range are eliminated (figure 2).

Immunohistochemical analysis of these animals has confirmed the dependence of the peptidergic population on NGF (Silos-Santiago *et al.* 1995). Thus CGRP and substance P are either vastly reduced or eliminated within the DRG and in the central projections of DRG neurons within the spinal cord (figure 3). An interesting feature of loss in these animals is that all small DRG neurons, even those which do not express TrkA in adulthood, are lost. Thus the population binding the lectin BSI-B4, which does not express TrkA in adulthood is totally eliminated in these animals (figure 3). As a consequence, the entire projection to the superficial dorsal horn appears to be lost as was previously reported in the animals deprived of NGF in an autoimmune paradigm (Ruit *et al.* 1992). Remaining DRG neurons in these animals appear to express NFH and either TrkB or TrkC. We can thus conclude that virtually all DRG neurons with a nociceptive phenotype are lost in these animals. It is also noteworthy that some neurons projecting to IIi which are depleted in these animals may have an innocuous mechanoreceptive phenotype (Molliver *et al.* 1995; Light 1992).

Results reported for the NGF−/− mutants are similar in many respects. Thus neuron loss in the lumbar DRGs and trigeminal ganglion is reported as 70% with virtually total loss of CGRP and SubP neurons and a only modest, if any, loss in heterozygote animals (see Crowley *et al.* 1994).

(*b*) *TrkB null mutants*

*Trk*B and BDNF (−/−) mutant animals have been less definitive with regards to dependence of DRG neuron subclasses on TrkB-signalling. As noted previously, the TrkB situation is complex in that there appear to be two populations of cells, a group expressing TrkB alone and others that express TrkB in conjunction with another Trk receptor. Many of these latter neurons appear to be co-localized with either the TrkA or TrkC population (McMahon *et al.* 1994). Cell counts in the DRG and trigeminal ganlion reported by most groups have suggested that the cell loss is approximately 30% in TrkB and BDNF null mutants with no loss reported in NT-4 mutants (Klein *et al.* 1993; Ernfors *et al.* 1994*a*; Jones *et al.* 1994; Conover *et al.* 1995; Liu *et al.* 1995). However, recent work suggests that neuron loss in DRGs may be less profound (I. Silos-Santiago *et al.*, unpublished observations). The identity of the classes of DRG neurons, if any, which are lost in these animals has not yet been established. It would seem likely from what has gone before that the TrkB population must be somehow represented in neurons projecting to deep layers of the dorsal horn because more dorsal and more ventral projections are eliminated in other knockout mice (see below). However, it is now clear that DRG neurons projecting to the deep dorsal horn are a very heterogeneous group of multiple functional types and may include neurons expressing all three Trks, either alone or in combination (see below). It is important to note in this regard that barrel formation is normal in these animals in both brainstem and somatosensory cortex (Henderson *et al.* 1995; see also Jones *et al.* 1994).

Interestingly, neuronal expression of the extracellular domain of TrkB is clearly present in some DRG neurons in *trk*B (−/−) animals, sugggesting that some neurons which express TrkB (I. Silos-Santiago & W. D. Snider, unpublished observations) are not dependent on TrkB signalling for survival. Presumably these are neurons in which TrkB is co-localized with another Trk. It is notewothy that BDNF is expressed within the DRG, particularly as development proceeds (Ernfors *et al.* 1992; Schecterson & Bothwell 1992; Apfel *et al.* 1995). It has been suggetsed that BDNF may be particularly important to approximately 30% of DRG neurons for survival in adulthood via an autocrine mechanism (Acheson *et al.* 1995).

(*c*) *TrkC null mutants*

The findings of DRG neuron loss in NT-3 and *trk*C null mutants have been definitive with regards to dependence of subpopulations of DRG neurons on NT-3/TrkC signalling. The most striking feature of both NT-3 and *trk*C null mutants is the appearance of writhing movements of the extremities that are noticeable within the first few days of life. Gross inspection in the *trk*C (−/−) animals reveals that the DRGs are of roughly normal size, whereas the dorsal roots are noticeably small. Examination of DRGs histologically in *trk*C (−/−) reveals a modest reduction in DRG neuron number of approximately 20% in lumbar DRGs, roughly the percentage of neurons which express TrkC in the adult mouse DRG (Klein *et al.* 1994). The dorsal roots are substantially depleted of large caliber axons, losing approximately 50% of the myelinated axons. Similarly, the dorsal columns are reduced by approximately 50% in area between the *trk*C (−/−) and the wild-type animals (figure 4).

A clue to the nature of the DRG neuron loss was provided by application DiI to the DRG allowing visualization of central axonal projections of DRG neurons (Klein *et al.* 1994). Remarkably, *trk*C(−/−) mice have no axons that extend into the ventral horn, or into the region of Clarke's column in the intermediate zone (figure 4). The results show that the collateral branches of Ia afferents which innervate the primary endings of muscle spindles in the periphery are completely eliminated. The total absence of ventral horn innervation is consistent with the idea that afferents innervating Golgi tendon organs and sencondary muscle spindle endings are also eliminated (see below). These classes of afferents are thought to be responsible for the sense of proprioception. Thus the strikingly abnormal limb movements of these animals are presumably due to loss of proprioceptive input. It is important that these axons never enter the spinal

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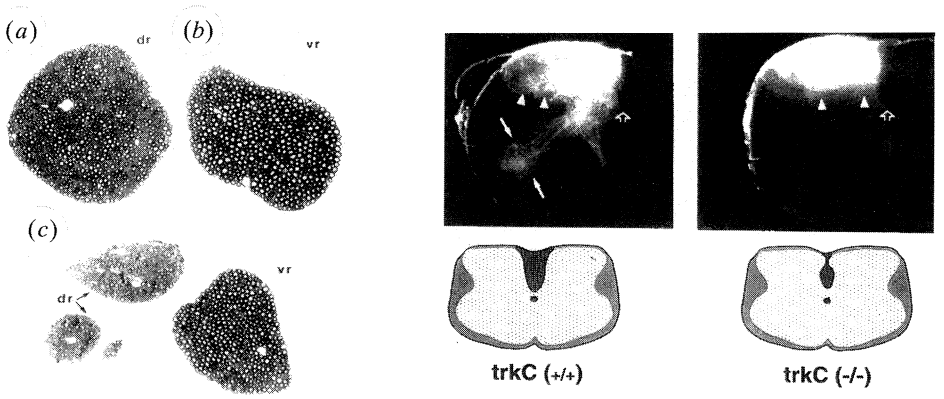

myelinated axons (Pn30)

	ventral roots	dorsal roots
trkC (+/+) ($n = 3$)	941 + 12	2176 + 194
trkC (–/–) ($n = 4$)	643 + 67	1150 + 85
t-static	4.01	9.61
p	0.01	>0.0001

area dorsal columns (Pn0)

	trkC (+/+)	trkC (-/-)
mean	99.861*	49.369*
s.d.	22.944	11.692
$n =$	38	66

* area in square microns
t-static: 14.87. significance: >0.0001.

Figure 4. Left panels: semi-thin sections of lumbar dorsal and ventral roots from PN30 *trk*C +/+ and −/− mice stained with toluidine blue. In the dorsal root, myelinated axon number was reduced by 50%. The numbers of axons in the ventral root were reduced by 28%. Right panels: Staining of the C5 dorsal root afferent projection to the spinal cord in P0 *trk*C+/+ and −/− mice. Note the total absence of projections to the ventral horn and the −/− mice. Also note that the dorsal columns in −/− animals are signficiantly reduced in area.

cord consistent with the idea that the parent neurons are lost at an early stage of development (Snider *et al.* 1994; Tessarollo *et al.* 1994). The proprioceptors are presumably the DRG neurons with the largest soma areas. It is important that TrkC-expressing neurons with intermediate size soma areas are also lost in these animals. Thus some classes of low-threshold mechanoreceptive neurons including those associated with Merkel cells and touch domes in the periphery are also dependent on NT-3/TrkC signalling (Albers *et al.* 1995; Lewin *et al.* 1995).

Work in NT-3(−/−) animals reveals similar findings with regards to proprioceptive neurons, although other DRG neuron classes are lost in these animals as well (see below). Additional information gained from analysis of serial, semithin sections of muscle in NT-3(−/−) mutants, is that peripheral end organs of proprioception, muscle spindles and Golgi tendon organs are also eliminated (Ernfors *et al.* 1994; Farinas *et al.* 1994). This observation confirms and generalizes earlier experiments that development of muscle spindles is critically regulated by innervating axons (for references to the primary literature, see Ernfors *et al.* 1994). It is important to point out that loss of DRG neurons is far more extensive in the NT-3 null mutants than in *trk*C null mutants. An attractive hypothesis which may explain discrepencies between DRG neuron loss in *trk*C and NT-3 null mutants is that NT-3 supports additional classes of neurons at very early developmental stages, possibly acting via TrkA (Farinas *et al.* 1994; and see below). The identitiy of the additional classes of afferents lost in NT-3 (−/−) animals has not yet been reported.

4. DEVELOPMENTAL CHANGES IN NEUROTROPHIN RECEPTOR EXPRESSION

(*a*) *TrkC down-regulation*

Work with neurotrophin and receptor null mutant mice published to date proves that most, if not all, DRG neurons pass through a period of dependence on a target-derived growth factor at some stage of development. It is important to note, however, that these results do not exclude survival-dependence on additional neurotrophin or non-neurotrophin neuronal growth factors in the embryonic or postnatal period. For example, no data yet speaks to whether DRG neurons which coexpress TrkB with another receptor require both BDNF (or NT-4) and NGF or NT-3, either sequentially or simultaneously during development. Certainly in the CNS, actions of multiple growth factors must be reuired to support neuronal survival as knockouts of single factors have not resulted in total elimination of particular populations.

In vitro studies have suggested the idea of sequential actions of neurotrophins during development. Thus sympathetic neuroblasts reuire NT-3 for survival *in vitro* whereas, at a later stage, sympathetic ganglion cells require NGF (Birren *et al.* 1993; Diccio-Bloom *et al.* 1993). Furthermore, trigeminal neurons change from dependence on BDNF or NT-3 to dependence on NGF around E13 (Buchman & Davies 1993). Such developmental switches are thought to be mediated by changes in neurotrophin receptor expression. This issue has been most carefully studied in sympathetic ganglia where TrkC is expressed early and is then down-regulated to be replaced by TrkA (Birren *et al.*

1993). Ernfors *et al.* (1992) have suggested that TrkC is also down-regulated during embryonic development in rat DRG. Our studies show that this down-regulation occurs between E11 and E13 in mice (White *et al.* 1995). It has been suggested that NT-3 functions to support sensory neurons before the arrival of sensory axons in their peripheral target fields (Buchman & Davies, 1993; see also Gaese *et al.* 1994). Neurotrophin and Trk null mutant mice provide ideal tools to test this hypothesis.

In preliminary studies in TrkA (−/−) mice, we have found massive neuron death in lower lumbar DRGs at embryonic day 13.5 (Silos Santiago *et al.* 1994; White *et al.* 1995). Virtually 50% of DRG neuronal profiles exhibit evidence of DNA fragmentation suggesting the very synchronous onset of DRG neuron death at this age. Axons of TrkA neurons in wild-type mice have extended substantial distance into hindlimb at E13 and have reached superficial ectoderm in proximal hindlimb. However, axons have not reached distal hindlimb at this age. Thus lower lumbar DRG neurons are clearly dependent on TrkA signalling before reaching definitive target fields. Both NGF and NT-3 are expressed in the hindlimb by E11, suggesting that either, or both molecules, might support DRG neurons at these early stages. There are, however, very important differences in expression patterns. NT-3 expression is diffuse, being abundant in ventral spinal cord and dermamyotome adjacent to the DRG, in muscle precursors in the hindlimb, as well as in superficial mesenchyme and deeper layers of developing skin. NGF expression, in contrast, is restricted to superficial mesenchyme and ectoderm, some distance from DRG cell bodies and axons projecting into the limb (White *et al.* 1995).

(*b*) *TrkA down-regulation*

Studies have not yet documented switches from dependence on neurotrophins to other non-neurotrophin growth factors, but such a change may well occur in DRG. In preliminary studies, we have shown that TrkA protein is expressed by 80% of mouse DRG neurons in the period between E15–PN1. TrkA expression is then downregulated in the first three postnatal weeks such that only 45% of DRG neurons express TrkA in adulthood (Molliver & Snider 1995). Lectin-binding of non-TrkA expressing small DRG neurons can first be demonstrated during this period of down-regulation of TrkA (Molliver & Snider 1995). This finding explains the discrepancy between percent of DRG neurons lost in TrkA (−/−) animals and percent of adult mouse DRG neurons which express TrkA. NGF is known to powerfully regulate substance P and CGRP in maturity in neurons which continue to express TrkA (Lindsay & Harmar 1989; see also Averill *et al.* 1995; Molliver *et al.* 1995) Presumably, downregulation of TrkA would abolish regulation of properties or functions of the non-TrkA-expressing neurons by NGF in maturity. A caveat associated with these findings is that these neurons might continue to express TrkA, but at levels below the limits of detection in histochemical procedures.

5. CONCLUSIONS

Differential expression of Trk receptor mRNA and protein by morphologically and cytochemically distinct classes of DRG neurons supports the idea that different functional classes of DRG neurons are regulated by different members of the neurotrophin family. Neurotrophin and neurotrophin receptor null mutant mice have confirmed this notion. Thus virtually all DRG neurons with morphological and cytochemical characteristics of nociceptors require NGF/TrkA signalling during development whereas all proprioceptors require NT3/TrkC signalling. Low-threshold mechanoreceptors appear to be a mixed population with at least some requiring NT-3/TrkC. Analysis of neuron survival in other peripheral ganglia including trigeminal strongly suggests that most, if not all peripheral ganglia are organized around differential survival-dependence of functionally distinct cell classes on target-derived neurotrophins. In addition to dependence on target-derived neurotrophins, neurons may depend on trophins acquired form other sources in their environment and acting via receptors which are subsequently down-regulated. Finally, some peripheral neurons may lose susceptibility to neurotrophins altogether outside the developmental period.

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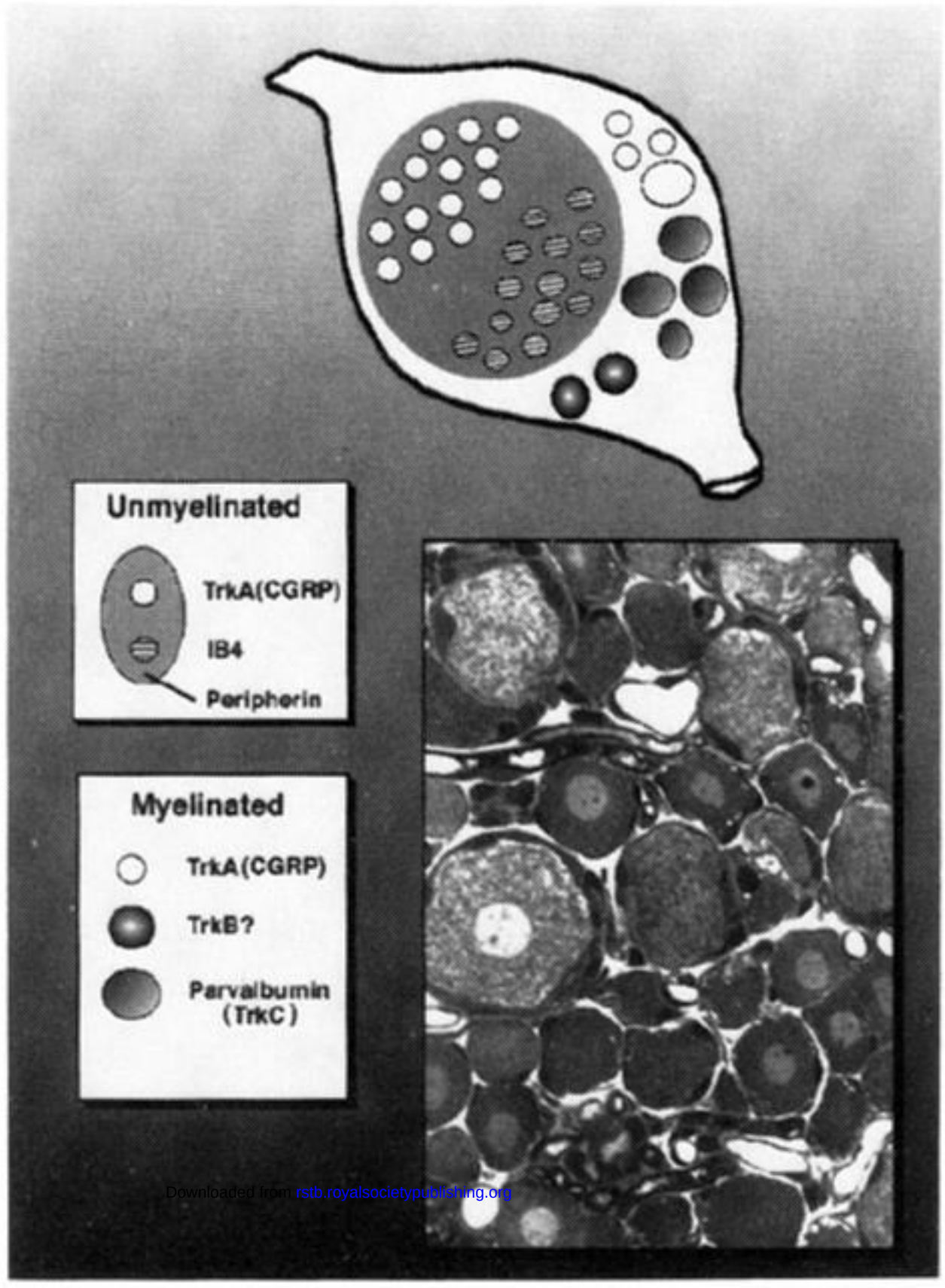
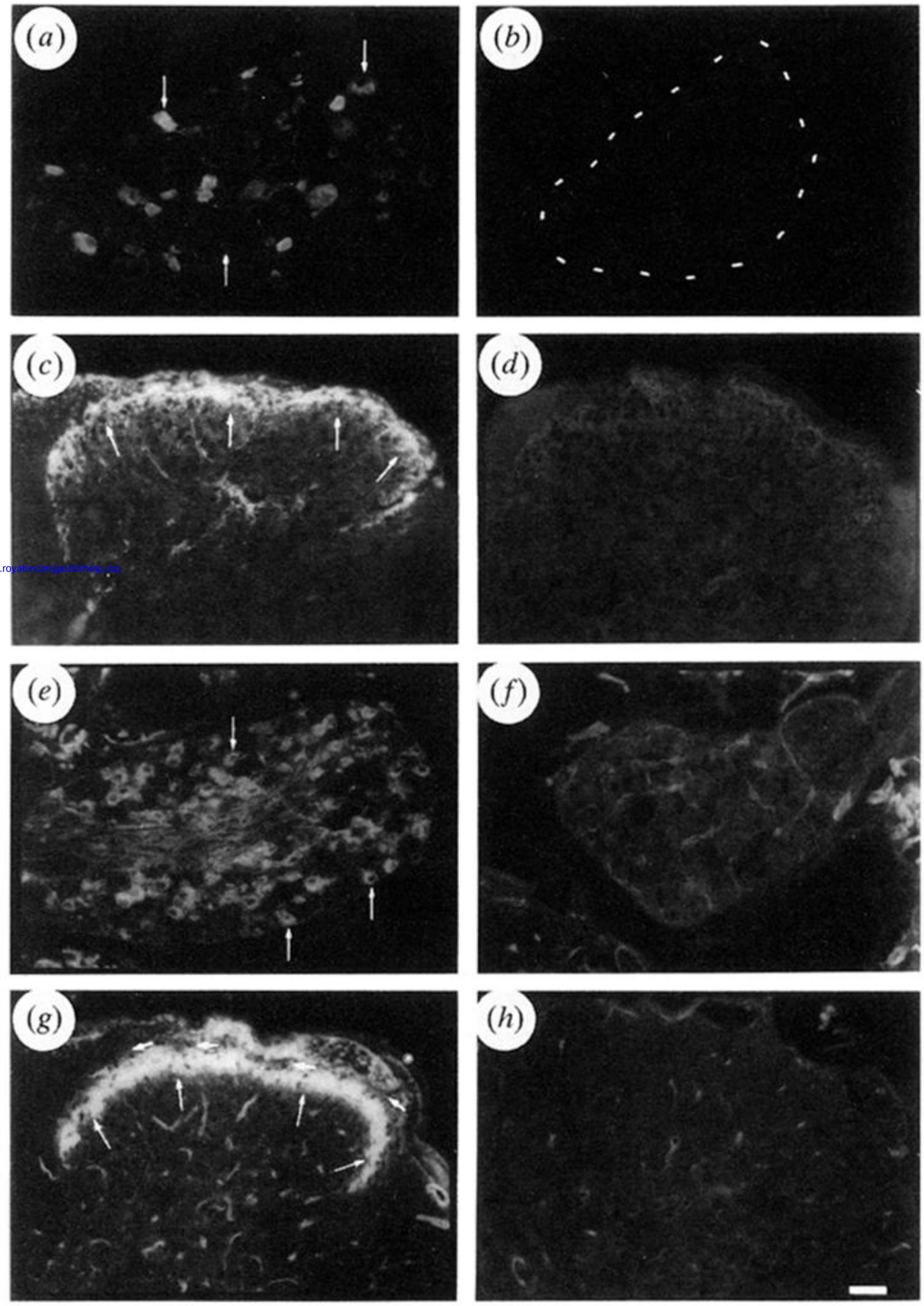
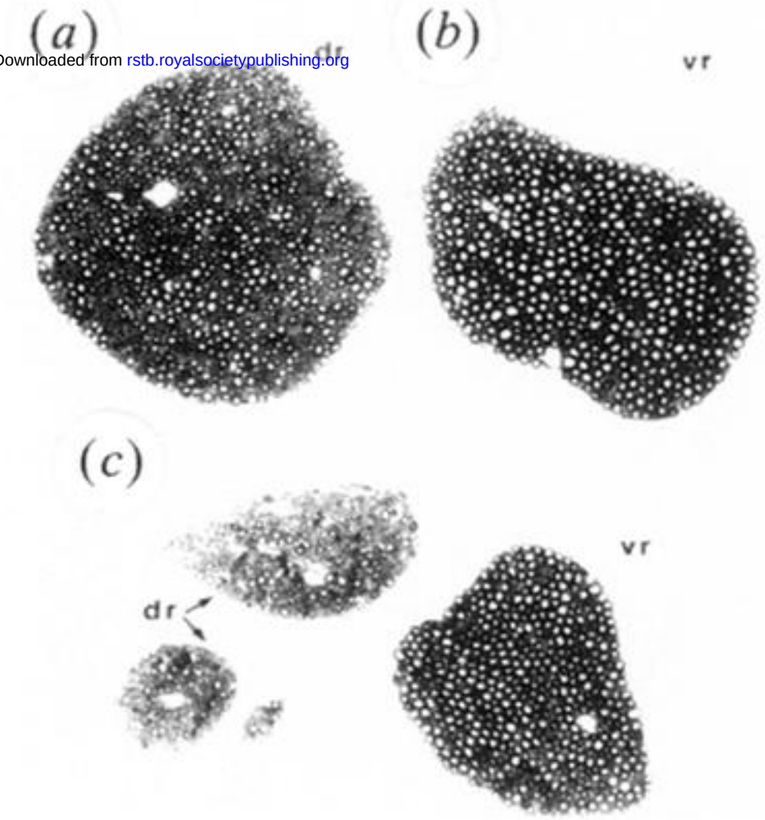
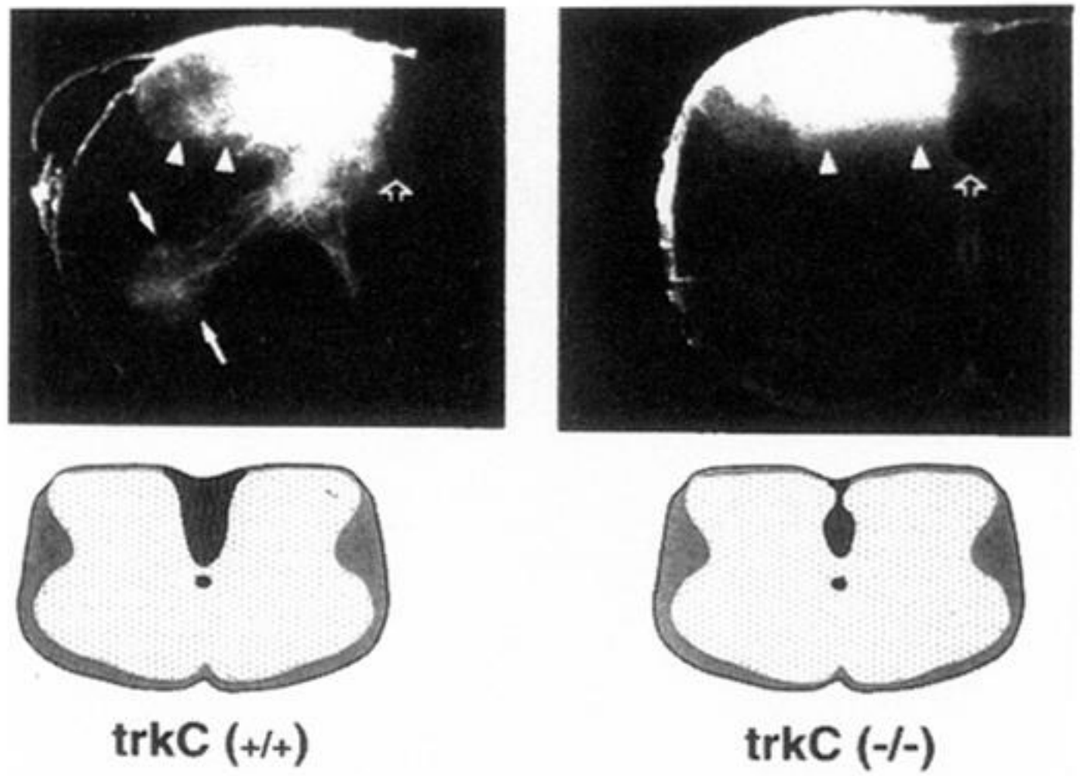


gure 1. Trk expression in adult rat DRG. Schematic of RG organization in terms of neurotrophin receptor pression. This scheme is for adult rat thoracic DRG. Inset ows large light (myelinated) and small dark (unmyelin-ed) neurons that have formed the traditional morphological assification of DRG neurons. Large light cells express the eavy chain of neurofilament in their cytoplasm whereas the nall dark neurons express the intermediate filament protein, eripherin. There appear to be four major classes of DRG eurons. Neurons expressing TrkA mRNA and protein present approximately 45% of DRG neurons. TrkA eurons have small and medium size soma cross-sectional reas. Most of these are unmyelinated, although a few yelinated (NFH expressing) DRG neurons are also labeled. he TrkA population is almost identical to the population of RG neurons which express CGRP. Note that TrkA does ot label the entire unmyelinated population. Non-TrkA nmyelinated neurons probably do not express any neuro-ophin receptor and are labelled by the lectin BSI-B4. eurons expressing TrkC represent 18% of adult rat DRG eurons and have large and medium-sized soma areas. A ubset of this population is labeled by the calcium binding rotein parvalbumin. Neurons expressing TrkB in isolation present approximately 5% of adult DRG neurons and are redominantly of intermediate size soma areas. Chemical arkers for the TrkB expressing population and smaller rkC neurons have not yet been identified.



gure 3. Both TrkA and non-TrkA expressing small DRG neurons are lost in TrkA −/− mice. Transverse sections DRGs and spinal cord in wild-type (left) and TrkA (−/−) mutant animals (right) illustrating immunoreactivity r CGRP and BS1. (*a*) DRGs from wild-type mice showing CGRP-IR neurons (arrows). (*b*) In trkA −/− mutants CGRP positive DRG neurons have been eliminated. The border of the DRG is shown in dashed lines. (*c*) CGRP mmunoreactivity (arrows) is present in the superficial laminae, I and II_o of the dorsal horn as well as in lamina 5. GRP is completely absent from the spinal cord of the −/− mutant. (*e*) BS1 labels a population of small DRG urons in wild-type mice. (*f*) All BS1 positive DRG neurons are absent in trkA −/− mice. (*g*) BS1 labels the entirety lamina II of the superficial dorsal horn (lower arrows). Lamina 1 is not stained (small upper arrows). (*h*) BS1 belling in spinal cord has also been eliminated in trkA −/− mutant mice. Scale bars 50 μM.





myelinated axons (Pn30)

	ventral roots	dorsal roots
trkC (+/+) ($n = 3$)	941 + 12	2176 + 194
trkC (–/–) ($n = 4$)	643 + 67	1150 + 85
t-static	4.01	9.61
p	0.01	>0.0001

area dorsal columns (Pn0)

	trkC (+/+)	trkC (-/-)
mean	99.861*	49.369*
s.d.	22.944	11.692
n =	38	66

* area in square microns
t-static: 14.87. significance: >0.0001.

gure 4. Left panels: semi-thin sections of lumbar dorsal and ventral roots from PN30 *trk*C +/+ and −/− mice ained with toluidine blue. In the dorsal root, myelinated axon number was reduced by 50 %. The numbers of axons the ventral root were reduced by 28 %. Right panels: Staining of the C5 dorsal root afferent projection to the spinal rd in P0 *trk*C+/+ and −/− mice. Note the total absence of projections to the ventral horn and the −/− mice. lso note that the dorsal columns in −/− animals are signficiantly reduced in area.