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IB4-Binding DRG Neurons Switch from NGF to GDNF Dependence in Early Postnatal Life

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Summary

We have tested the role of glial cell line-derived neurotrophic factor (GDNF) in regulating a group of putatively nociceptive dorsal root ganglion (DRG) neurons that do not express calcitonin gene-related peptide (CGRP) and that downregulate the nerve growth factor (NGF) receptor tyrosine kinase, TrkA, after birth. We show that mRNA and protein for the GDNF receptor tyrosine kinase, Ret, are expressed in the DRG in patterns that differ markedly from those of any of the neurotrophin receptors. Most strikingly, a population of small neurons initiates expression of Ret between embryonic day 15.5 and postnatal day 7.5 and maintains Ret expression into adulthood. These Ret-expressing small neurons are selectively labeled by the lectin IB4 and project to lamina IIi of the dorsal horn. Ret-expressing neurons also express the glycosyl-phosphatidyl inositol-linked (GPI-linked) GDNF binding component GDNFR-α and retrogradely transport ^{125}I-GDNF, indicating the presence of a biologically active GDNF receptor complex. In vitro, GDNF supports the survival of small neurons that express Ret and bind IB4 while failing to support the survival of neurons expressing TrkA and CGRP. Together, our findings suggest that IB4-binding neurons switch from dependence on NGF in embryonic life to dependence on GDNF in postnatal life and are likely regulated by GDNF in maturity.

Introduction

A fundamental advance in the study of somatosensory systems has been the discovery of molecules that regulate the development of primary sensory neurons. The most extensively studied of these are members of the neurotrophin family of nerve growth factor (NGF) related molecules, all of which have powerful regulatory effects on primary sensory neurons (reviewed by Fariñas and Reichardt, 1996; Lewin and Barde, 1996; Snider and Silos-Santiago, 1996; Snider and Wright, 1996). The different members of the neurotrophin family have actions that are specific for subsets of neurons that mediate different sensory modalities. Specificity is determined both by restricted patterns of neurotrophin expression in target tissues of sensory axons and by cell type-specific neuronal expression of different members of the Trk family of receptor tyrosine kinases, which mediate neurotrophin actions. Most classes of primary sensory neurons studied to date, including nociceptors, proprioceptors, auditory, cutaneous, and visceral mechanoreceptors, require one or more members of the neurotrophin family for survival during development (Klein, 1994; Ernfors et al., 1995).

In addition to their critical role in supporting the survival of sensory neurons during development, neurotrophins also regulate physiological properties of mature sensory neurons (Lewin and Barde, 1996). The most extensively studied example of this phenomenon is the action of NGF on nociceptors. In adults, the NGF receptor tyrosine kinase, TrkA, is extensively colocalized with calcitonin gene-related peptide (CGRP), a neuropeptide associated with nociceptive signaling (Averill et al., 1995). Many of these neurons also express the nociceptive neuropeptide substance P. NGF levels regulate functional properties of nociceptive neurons, including levels of neuropeptide expression, nociceptive thresholds, and neuronal responsiveness to capsaicin (Lewin et al., 1994; McMahon et al., 1995; reviewed by McMahon, 1996; Woolf, 1996).

Roughly 80% of DRG neurons in mouse and rat require NGF for survival during embryonic development, including all small neurons (Ruit et al., 1992; Silos-Santiago et al., 1995). However, half of these small neurons subsequently downregulate TrkA during the first 3 weeks after birth and express no member of the Trk family or p75 in maturity (Bennett et al., 1996; Molliver and Snider, 1997). The downregulation of TrkA occurs selectively in a population that does not express CGRP or substance P but is labeled by the plant lectin IB4, resulting in the presence of a substantial population of unmyelinated neurons that do not express the NGF receptor and presumably are not regulated by NGF in maturity (Bennett et al., 1996; Molliver and Snider, 1997). Interestingly, whereas TrkA-expressing neurons project to lamina I and the outer region of lamina II (lamina IIo) of the spinal cord, IB4-binding neurons project principally to the interior of lamina II (lamina IIi), suggesting that information conveyed by these two populations may be processed differently in the CNS (Hunt and Rossi, 1985; Light, 1992; Molliver et al., 1995).

The powerful effects of neurotrophins on primary sensory neurons make it logical to inquire whether a non-neurotrophin growth factor may regulate the DRG neurons which express no Trk family member in maturity. One promising candidate is glial cell line-derived neurotrophic factor (GDNF), a member of the TGFβ superfamily (Lin et al., 1993). This molecule appears to be the prototype of a family of growth factors that includes a second recently characterized molecule, neurturin (Kotz-

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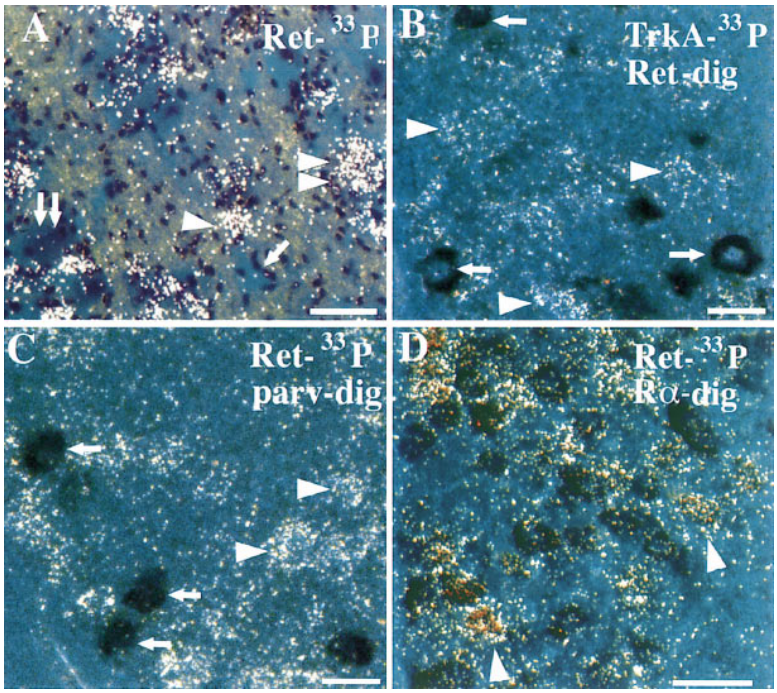

Figure 1. Ret mRNA Is Not Coexpressed with mRNA for TrkA or Parvalbumin

(A) Adult mouse lumbar DRG section hybridized with a Ret antisense riboprobe. Many neurons, including both small (arrowhead) and large (double arrowhead) ones, express Ret mRNA. However, both small (arrow) and large (double arrow) neurons are negative for Ret mRNA.

(B) Section hybridized simultaneously with a TrkA isotopic probe and Ret colorimetric probe. Arrows show examples of Ret-positive, TrkA-negative neurons; arrowheads indicate TrkA-positive, Ret-negative neurons. Virtually no overlap exists between the Ret and TrkA populations.

(C) Section hybridized simultaneously with a Ret isotopic probe (arrowheads) and parvalbumin colorimetric probe (arrows). Virtually no overlap exists between the Ret and parvalbumin populations.

(D) DRG section hybridized simultaneously with a Ret isotopic probe and a colorimetric GDNFR-α probe. Most Ret neurons also coexpress GDNFR-α (arrowheads). Scale bars, 50 μm.

bauer et al., 1996). GDNF supports in vitro and in vivo survival of a number of neuronal classes, including motor neurons, mesencephalic dopaminergic neurons, and enteric ganglion cells (reviewed by Unsicker, 1996; Lapchak et al., 1996). The receptor tyrosine kinase Ret has recently been identified as a signaling receptor for GDNF and neurturin and acts in concert with one of two recently identified glycosyl-phosphatidyl inositol-linked (GPI-linked) proteins, GDNFR-α, also referred to as TrnR1, and a homologous molecule variously referred to as GDNFR-β, TrnR2, or NTNRα (Durbec et al., 1996; Jing et al., 1996; Treanor et al., 1996; Trupp et al., 1996; Baloh et al., 1997; Buj-Bello et al., 1997; Creedon et al., 1997; Klein et al., 1997). Ret is expressed in the DRG as early as E13, and expression persists into adulthood (Pachnis et al., 1993; Treanor et al., 1996). Furthermore, both GDNF and neurturin support sensory neuron survival in vitro (Buj-Bello et al., 1995; Kotzbauer et al., 1996; Matheson et al., 1997). Finally, GDNF can largely prevent the loss of DRG neurons occurring after neonatal axotomy (Matheson et al., 1997). These results, taken together, suggest that GDNF and/or neurturin influence DRG development but do not provide information about whether these trophic factors regulate specific subpopulations of sensory neurons.

To test the idea that sensory neurons downregulating TrkA may be subsequently regulated by GDNF, we examined the distribution of Ret mRNA and protein in histochemically identified subsets of DRG neurons and assessed the effects of GDNF on the survival of identified populations in vitro. We show that Ret mRNA and protein are expressed by specific subpopulations of adult DRG neurons that include IB4-binding but not TrkA-expressing neurons. Interestingly, Ret expression in small neurons is not initiated until shortly before the downregulation of TrkA. Finally, GDNF selectively supports IB4-binding neurons from neonatal DRGs in vitro. Together, our findings suggest that IB4-binding DRG neurons switch from NGF to GDNF dependence in early postnatal life.

Results

IB4-Binding DRG Neurons Selectively Express Ret mRNA and Protein

In order to explore patterns of Ret expression in the DRG, we first cloned a Ret cDNA which was used to generate a cRNA probe for in situ hybridization. Analysis of Ret hybridization patterns in adult mouse L5 DRG sections revealed that ~60% of DRG neurons expressed Ret mRNA (Figure 1A). Ret mRNA did not appear to be segregated on the basis of soma size, as both small (arrowheads) and large (double arrowheads) neurons expressed Ret. Interestingly, there were also both small (arrows) and large (double arrows) neurons that lacked expression of Ret mRNA.

In order to determine the extent to which NGF-responsive neurons expressed Ret, we used a combination of isotopic and colorimetric probes to identify neurons that expressed mRNAs for Ret and TrkA (Figure 1B). Most TrkA neurons were small (arrowheads) and therefore provided a good candidate for overlap with small neurons that expressed Ret (arrows). However, expression of Ret and TrkA mRNAs in L5 DRG showed very little overlap; of 1629 Ret-expressing neurons, only 82 (5%) also expressed TrkA mRNA (Table 1). These findings suggest that GDNF and NGF influence separate populations of DRG neurons in the adult.

Because a subset of very large neurons also expressed Ret, adult DRG sections were hybridized for mRNAs encoding Ret and parvalbumin, a marker for large neurons that innervate muscle spindles (Ia afferents; see Celio, 1990; Copray et al., 1994; Ernfors et al., 1994; Figure 1C). Almost no overlap was seen between

Table 1. Colocalization of Ret mRNA with TrkA, Parvalbumin, and GDNFR-α

	Ret+ cells (N)	Double-labeled cells	Ret population (%)
TrkA	1629	82	5.0
Parvalbumin	1970	22	0.1
GDNFR-α	1147	1016	88.6

The extent of colocalization of Ret mRNA with TrkA, parvalbumin, and GDNFR-α mRNA was determined by quantifying neurons double hybridized with isotopic and colorimetric riboprobes in adult L5 DRG.

neurons that expressed Ret mRNA (arrows) and parvalbumin mRNA (arrowheads; 22 of 1970 neurons [0.1%]), indicating that large Ret neurons are not members of the Ia afferent population (Table 1). These findings indicate that GDNF is unlikely to regulate proprioceptors.

Functional GDNF receptor requires the expression of Ret in combination with at least one GPI-linked protein, GDNFR-α. Similar to the pattern of Ret-expressing neurons, hybridization with a probe for mouse GDNFR-α yielded labeling of many DRG neurons. Dual in situ hybridization for Ret and GDNFR-α mRNAs (Figure 1D) revealed extensive colocalization of these two GDNF receptor mRNAs in DRG neurons (arrowheads); 89% of Ret-expressing neurons expressed mRNA encoding GDNFR-α (Table 1). The fact that a small number of Ret-positive neurons were not positive for GDNFR-α may be due to differences in the relative sensitivity of colorimetric versus isotopic labeling. Alternatively, non-GDNFR-α expressing neurons may express another GDNF or neurturin-binding GPI-linked molecule.

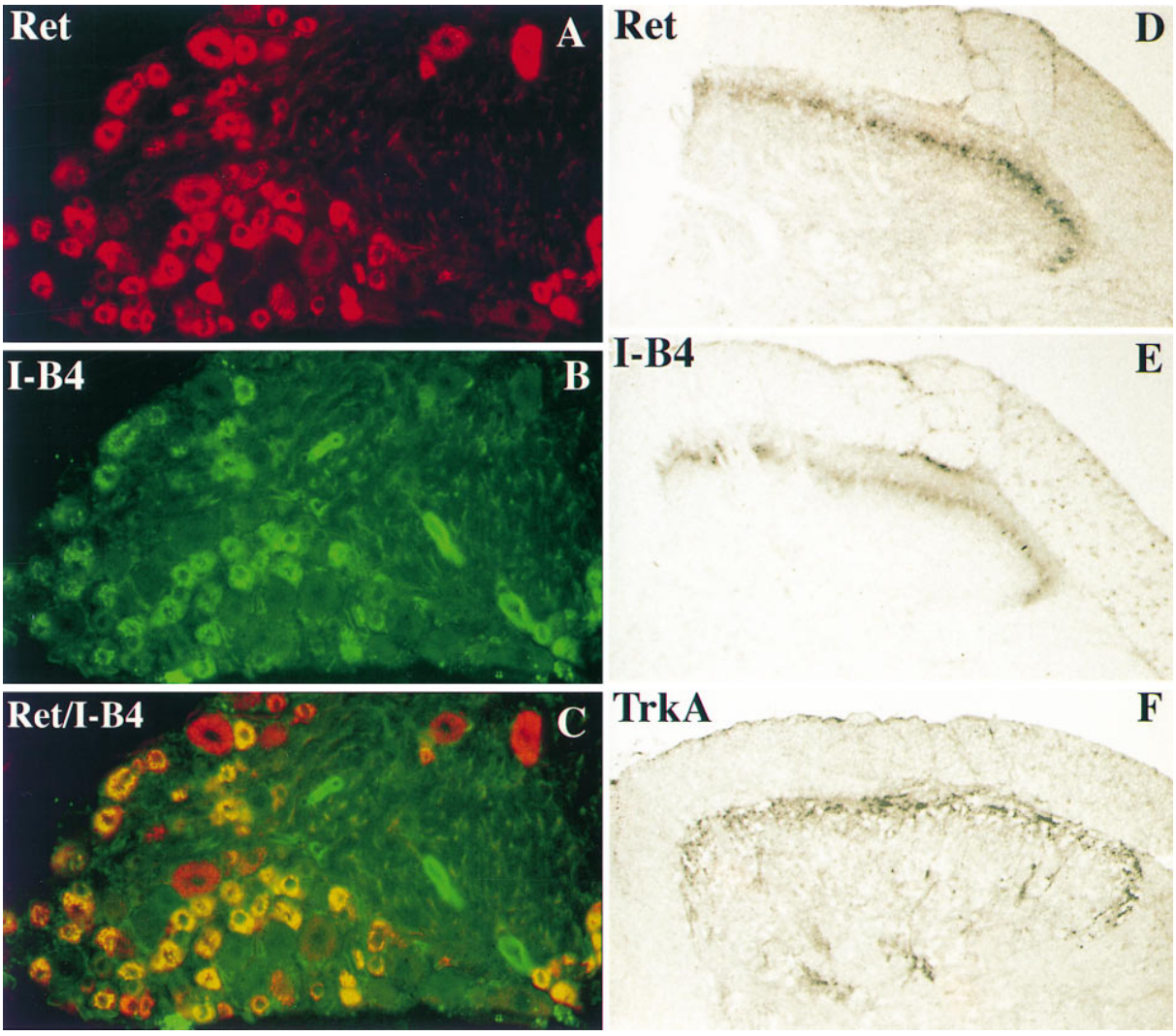

Figure 2. Ret Is Selectively Expressed in the Adult by IB4-Binding Neurons that Project to the Interior of Lamina II

Panels (A) through (C) depict fluorescent immunocytochemical labeling of Ret (red; [A]), IB4 (green; [B]), and the same two frames digitally superimposed to reveal the extent of colocalization (C). Yellow indicates double-labeling. Note that all IB4-binding neurons are small in diameter and coexpress Ret. Panels (D) through (F) compare the distribution of Ret (D), IB4 (E), and TrkA-positive (F) axon terminals in adjacent sections of adult dorsal horn. Ret and IB4 are both tightly restricted to the interior of lamina II, whereas TrkA staining includes laminae I and the outer region of lamina II. A few TrkA-positive axons are also observed in lamina V.

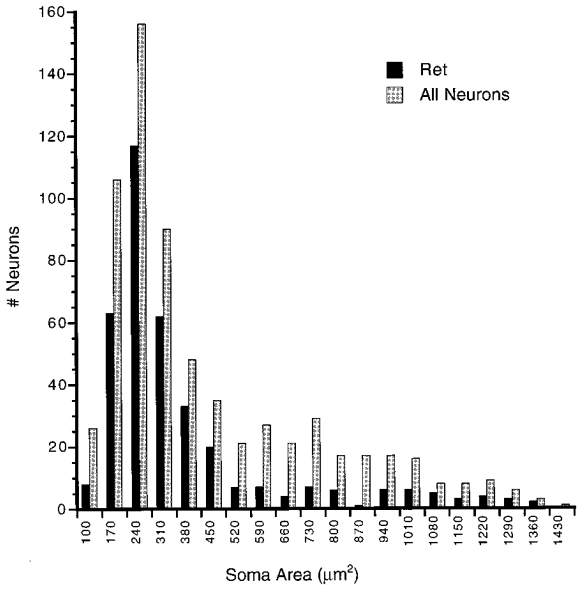

Figure 3. Size Distribution of Ret-IR Neurons in Adult Mouse

Size frequency histograms of Ret-positive and all mouse L5 DRG neurons. Although the majority of Ret-positive neurons are small, roughly half of the large neurons and some intermediate-sized neurons also express Ret.

In order to further characterize the identity of Ret-expressing neurons, we generated a polyclonal antibody to Ret and studied the distribution of Ret protein in mouse DRG and spinal cord. Immunocytochemical staining for Ret in sections of adult L5 DRG revealed robust punctate staining with a distribution very similar to that of Ret mRNA (Figure 2A). Ret-immunoreactive (Ret-IR) neurons comprised 61.9% ± 1.3% of DRG neurons and were of all sizes (Figure 3). Although the majority were small in diameter, roughly half of the largest cells in the ganglion were also Ret-IR.

Given the lack of colocalization of Ret mRNA with TrkA mRNA, staining with the Ret antibody was combined with labeling by the lectin IB4, a known marker of non-TrkA expressing unmyelinated DRG neurons (Averill et al., 1995; Molliver et al., 1995; Figure 2B). Within the subset of small Ret-IR neurons, nearly all were labeled by IB4 (Figure 2C, yellow cells; Table 2). Although IB4-negative (primarily large) Ret-IR neurons were frequently seen (Figure 2C, orange cells), IB4-positive Ret-negative neurons were not seen in any of the sections examined.

The laminar specificity of primary afferent termination in the spinal cord grey matter can provide important insight into the functional identity of the primary afferent neurons. Therefore, the distribution of Ret-IR axons was examined in sections of lumbar spinal cord. Consistent with the extensive overlap of Ret and IB4 staining, Ret immunoreactivity was restricted to a dense band in the superficial dorsal horn corresponding to lamina IIi (Figure 2D). This pattern was identical to that of IB4 staining (Figure 2E) but differed from the pattern exhibited by TrkA immunoreactivity, which labeled axons that project to laminae I and IIo (Figure 2F). Perhaps surprisingly, no evidence of projections into the deeper dorsal horn or ventral horn was observed in adult spinal cord that could be attributed to axons of the large diameter Ret-positive neurons.

Table 2. Colocalization of IB4 Binding and Ret Immunoreactivity

Ret+ cells (N)	IB4+ cells (N)	Ret w/IB4 (%)	IB4 w/Ret (%)
367	230	62.7	100

The colocalization of Ret and IB4 staining was quantified in double-labeled sections of L5 DRGs from three adult mice.

Ret-Expressing Neurons Show Specific Retrograde Transport of ^{125}I-GDNF

To determine whether labeling for Ret mRNA and protein represented biologically active GDNF receptor in the adult, ^{125}I-GDNF was injected into rat sciatic nerve and the distribution of labeled GDNF was examined in the L4/L5 DRGs by combining autoradiography with immunocytochemistry for Ret, TrkA, or α-internexin, a neurofilament protein restricted to non-TrkA expressing small neurons in the adult DRG (Molliver et al., 1995). Examination of neurons that retrogradely transported ^{125}I-GDNF revealed a neuronal population with a size distribution similar to that demonstrated in the mouse with Ret mRNA (data not shown). Roughly 50% of Ret-IR neurons showed GDNF uptake (Figures 4A and 4B, open arrowheads). That not all Ret-IR neurons transported GDNF (Figure 4B, asterisk) may be explained by the fact that not all neurons in the L4/L5 DRGs project via the sciatic nerve. However, in no instances were Ret-negative cells labeled by ^{125}I-GDNF uptake (Table 3). Many small neurons that transported ^{125}I-GDNF were α-internexin-IR, consistent with the colocalization of Ret-IR and IB4 (Figure 4C, open arrowheads; Table 3). Furthermore, few TrkA-IR neurons (Figure 4D, asterisks) transported ^{125}I-GDNF, consistent with the limited colocalization of Ret and TrkA (Table 3). Thus, subsets of neurons that expressed Ret mRNA and protein also exhibited retrograde transport of ^{125}I-GDNF from the sciatic nerve, indicating specific GDNF receptor activity.

IB4-Binding Sensory Neurons Upregulate Ret Expression in Late Embryonic Life

To examine the developmental expression of Ret in the DRG, mouse lower lumbar DRGs of developmental ages E11.5–E15.5 were hybridized to identify neurons expressing Ret mRNA. Similarly, Ret-IR was visualized in lower lumbar DRGs from E13 to postnatal day (PN) 7. Striking changes in the distribution of Ret were observed between embryonic and early postnatal development. At E11.5, a small subpopulation of neurons already expressed Ret mRNA (Figure 5A). At E13.5 and E15.5, the percentage of neurons expressing Ret was roughly similar to that observed at E11.5 (Figures 5B and 5C). This embryonic pattern in the DRG concurs with initial studies of Ret expression in the mouse at E13.5 (Pachnis et al., 1993). Further, it was possible in sections of E15.5 DRG to discern that Ret-expressing neurons were larger than most non-Ret expressing neurons (see below). In addition, silver grains were detectable over the developing motor pools at every age examined (arrows).

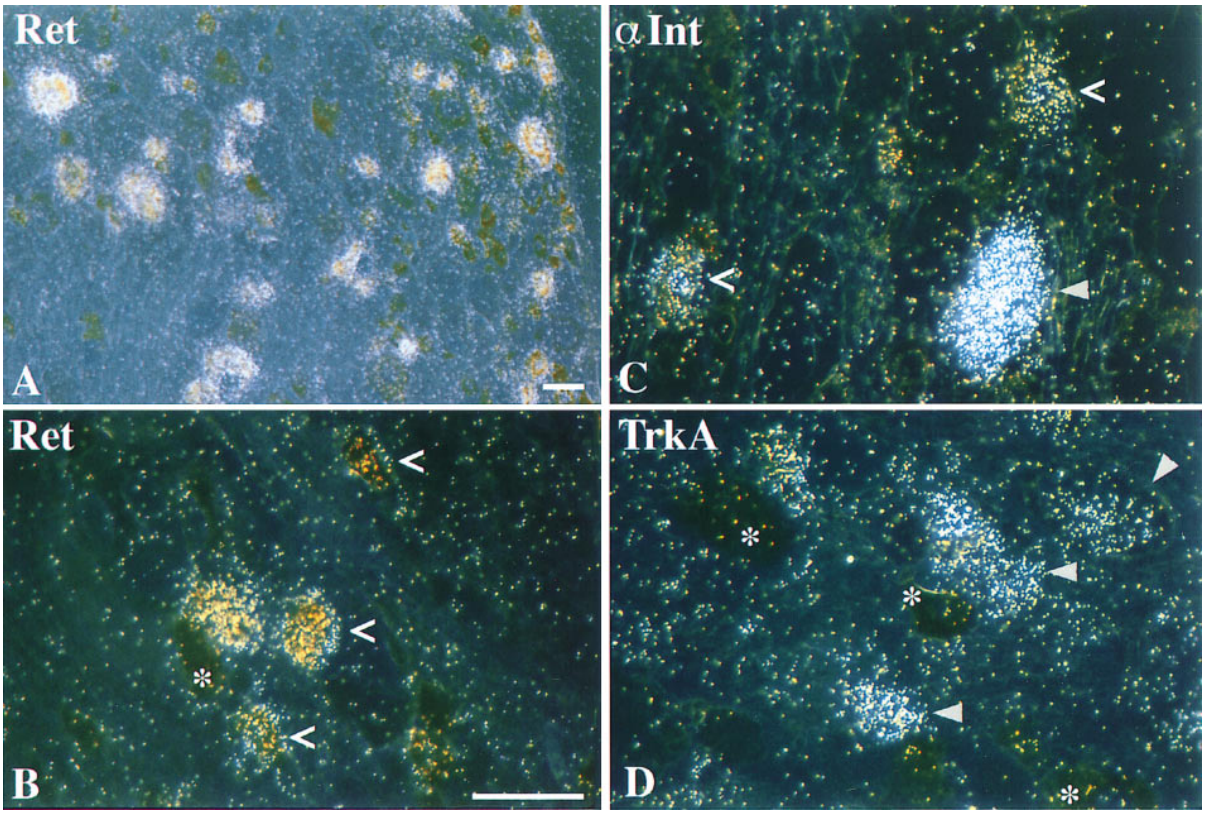

Figure 4. GDNF Is Retrogradely Transported by Ret-Immunoreactive Neurons

Photomicrographs of adult rat L5 DRG sections illustrating the overlap between ^{125}I-GDNF autoradiography, representing uptake of ^{125}I-GDNF from the sciatic nerve, and immunocytochemistry for Ret, α-internexin, or TrkA.

(A) Low-power view showing that ^{125}I-GDNF-positive cells are also stained for Ret, indicating that adult Ret-expressing neurons are capable of retrogradely transporting GDNF to the cell body.

(B) A similar section shown at high power to illustrate the colocalization of Ret staining with ^{125}I-GDNF uptake. Open arrowheads indicate several double-labeled neurons; asterisk labels a Ret-IR neuron that did not transport ^{125}I-GDNF.

(C) High-power photograph of labeling for ^{125}I-GDNF and α-internexin, a neurofilament protein expressed by small neurons lacking TrkA. Open arrowheads point out examples of the extensive overlap of ^{125}I-GDNF and α-internexin in small cells; solid arrowhead indicates a typical large ^{125}I-GDNF-labeled neuron negative for α-internexin.

(D) High-power photograph of ^{125}I-GDNF and TrkA labeling. Very little overlap exists between neurons that transport ^{125}I-GDNF and are TrkA-IR. Arrowheads point out TrkA-negative, ^{125}I-GDNF-labeled neurons; asterisks indicate TrkA-positive, ^{125}I-GDNF-negative neurons. Scale bars, 50 μm; scale bar in (B) is valid for (B), (C), and (D).

Immunocytochemistry for Ret protein in E13.5 DRG sections identified only a few neurons. At E15.5, more cells were labeled, but Ret-IR cells represented <15% of DRG cells overall (Figure 5E). Size frequency analysis revealed that Ret expression was restricted to a subset of large neurons. The mean soma area of Ret-IR neurons at this age was 187.8 ± 7.6 μm^2. In contrast, TrkA-IR neurons at this age included all of the small neurons and some neurons of intermediate size, with a mean soma area of 80.6 ± 1.8 μm^2 (Figure 6A). At E17.5, a few small Ret-IR neurons were visible (data not shown). After E17, the number of small Ret-IR neurons continued to increase with the age of the animal (Figures 5F, 5G, and 6B). By PN7.5, the pattern and percentage of Ret-IR neurons was similar to adults (Figures 5G, 5H, and 6B). In contrast to the developmental pattern for Ret, the percentage of TrkA-expressing cells declined steadily between P1 and P21 (Figure 6B; see Molliver and Snider, 1997).

Table 3. Colocalization of ^{125}I-GDNF Retrograde Transport with Ret, TrkA, and α-internexin

	GDNF+ cells labeled (N)	GDNF+ cells labeled
Ret	258/258	100
TrkA	53/408	13
α-internexin	172/409	42

Quantitation of overlap between neurons immunoreactive for Ret, TrkA, or α-internexin and neurons labeled by ^{125}I-GDNF autoradiography (GDNF+), in sections from adult L4 and L5 DRGs.

Large-caliber, Ret-IR afferent projections penetrating the deeper regions of the spinal cord were clearly visible in the developing embryo. These large Ret-IR afferents were most densely labeled at E17.5 and were localized in laminae III and IV, the primary target fields of cutaneous low threshold mechanoreceptors (Figure 7A). Why these projections were not immunoreactive in older animals is unclear. In contrast, fine caliber afferents projecting to the superficial dorsal horn were faintly visible

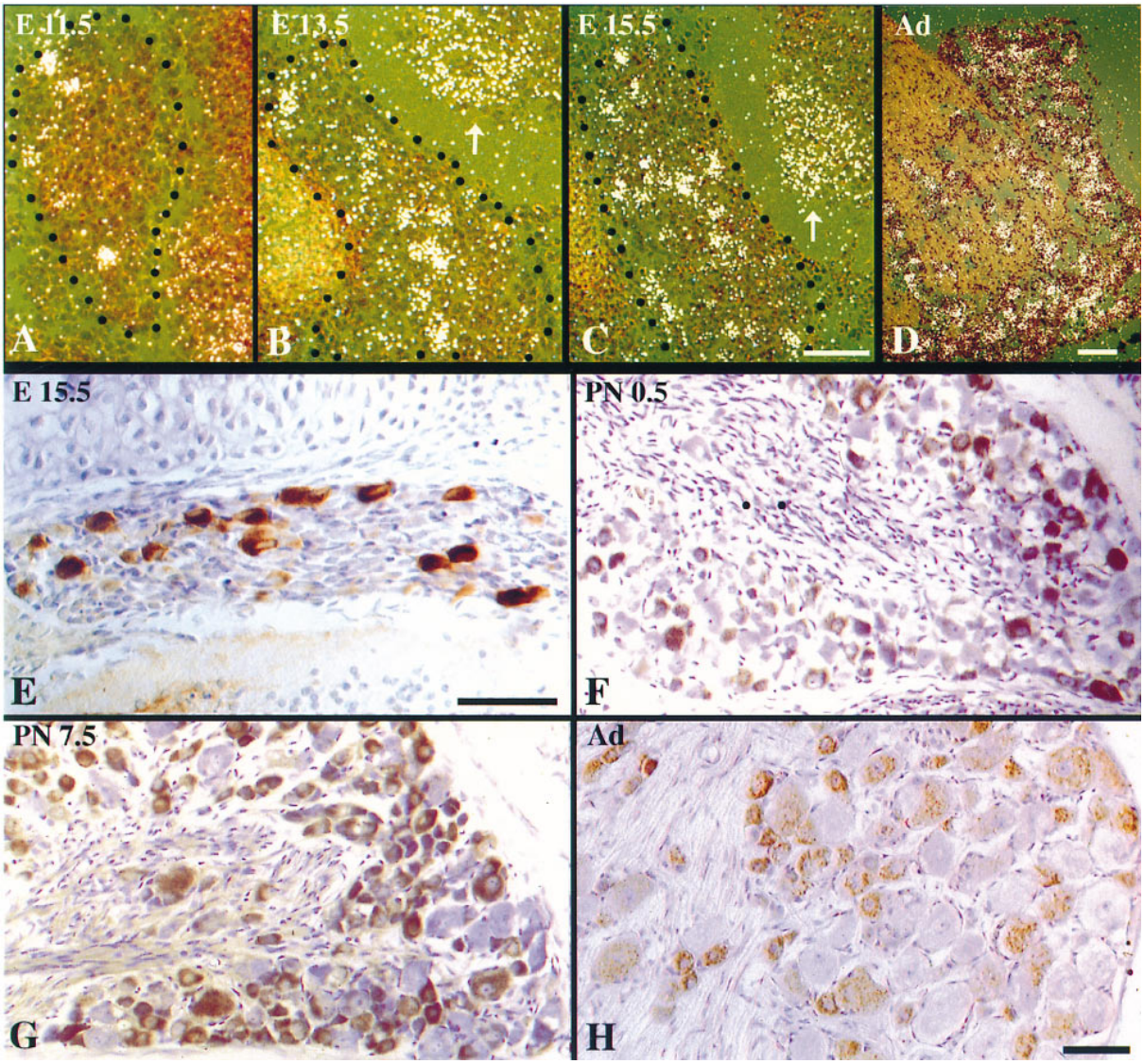

Figure 5. Ret Is Upregulated by Small Neurons during Development

(A–D) Mouse DRG sections at E11.5 (A), E13.5 (B), and E15.5 (C) showing the distribution of Ret mRNA in embryonic DRG neurons. Ret message is detectable as early as E11.5 in a small number of neurons, and this pattern is maintained at E13.5 and E15.5. At E15.5, it is clear that Ret-expressing neurons are a population of large-sized neurons. In contrast, Ret mRNA is expressed by many more neurons in the adult (D). Arrows in (B) and (C) point out Ret expression in the ventral motor pool.

(E–H) Ret immunocytochemistry in DRG sections from E15.5 (E), PN0.5 (F), PN7.5 (G), and adult (H) animals illustrating the progressive increase in the number of Ret-IR neurons during late embryonic and early postnatal development. Similar to the localization of mRNA, Ret immunocytochemistry only labels a subset of large neurons at E15.5, but labels progressively more small neurons between E15.5 and P7.5, at which point the distribution appears mature. Black dots outline the DRG. Scale bars, 50 μm; scale bar in (H) is valid for (F), (G), and (H).

at E17.5 and were densely stained at PN7.5 and in adult. In the periphery, many Ret-IR axons were visible in the dermis and were often associated with developing hair follicles (Figure 7B).

GDNF Selectively Supports IB4-Binding Neurons In Vitro

In order to ask whether GDNF supports the survival of subpopulations of sensory neurons, we tested the ability of GDNF to support dissociated murine DRG neurons plated at PN0 on laminin and maintained over a period of 2 weeks. Further, we determined which classes of neurons are supported by GDNF by using immunocytochemistry for TrkA and CGRP to identify the peptidergic neurons and for IB4 and Ret to identify the nonpeptidergic neurons (Figure 8).

There was a baseline level of neuronal survival after 2 weeks in vitro, even in the absence of any added growth factors. Some survival in the absence of growth factors was expected, as DRG neurons undergo a marked change in NGF dependence in neonatal life (Yip et al., 1984; Lindsay, 1988; Lewin and Mendell, 1993). Furthermore, nonneuronal cells in these cultures may have synthesized survival-promoting factors. However,

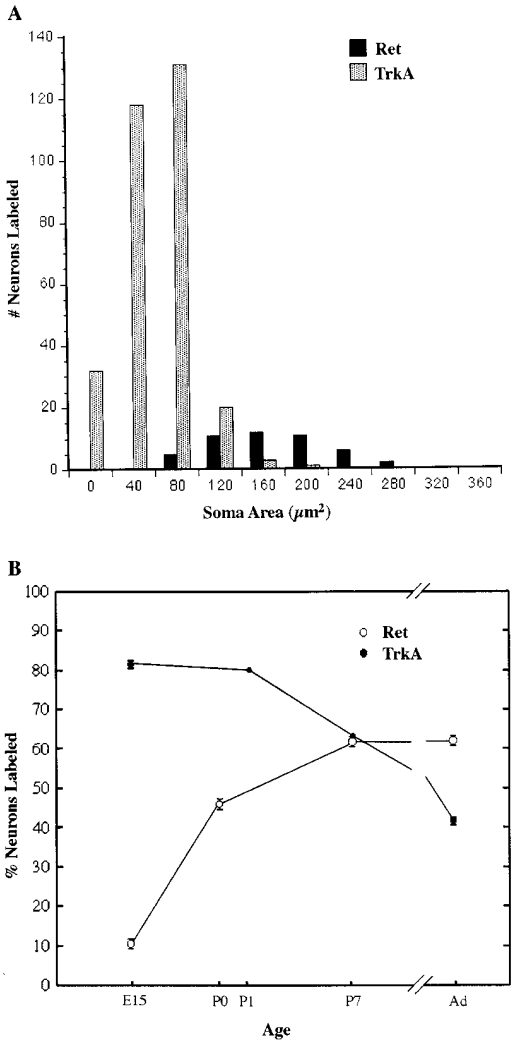

Figure 6. Changes in Expression of Ret and TrkA in DRG Neurons during Development

(A) Size frequency histogram comparing the distribution of Ret- and TrkA-positive neurons in E15.5 lower lumbar DRGs. At this age, Ret is exclusively expressed by large diameter neurons, whereas TrkA-positive neurons include all of the small neurons and some intermediate neurons.

(B) This graph illustrates the temporal correlation between the progressive increase in the percentage of Ret-expressing neurons and the decrease in the percentage of TrkA-expressing neurons during development. The percentage of Ret-positive small neurons steadily increases after E15.5 and reaches mature levels at PN7.5, while TrkA is selectively downregulated in small neurons after PN0.5 and acquires a mature distribution pattern between PN14.5 and PN21.5. Percentages of Ret-positive cells based on counts of at least 600 neurons in lumbar DRGs from each of three mice. TrkA data taken from Molliver and Snider, 1997. Error bars, SEM (some error bars obscured by symbols).

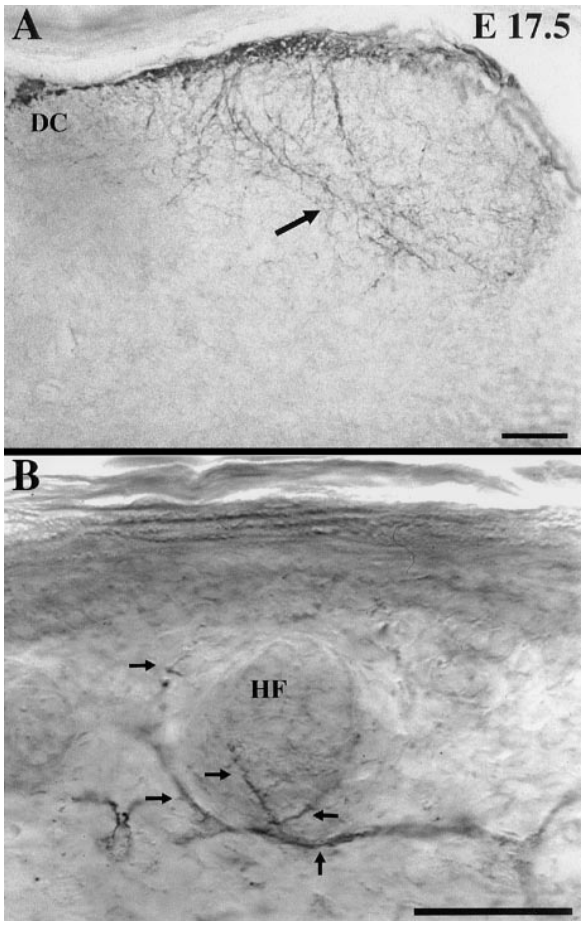

Figure 7. Axonal Projections of Developing Ret-Positive DRG Neurons

(A) At E17.5, central projections of large-caliber Ret-positive axons are visible penetrating laminae III–IV of the deep dorsal horn (arrow), the major target region of cutaneous low threshold mechanoreceptors. Left border of the photograph is at the midline, dorsal columns are labeled "DC". Scale bar, 200 μm.

(B) High power photomicrograph of Ret-positive axon branches (arrows) in close association with a developing hair follicle (HF) in the dermis. Scale bar, 50 μm.

the addition of GDNF to the cultures supported the survival of more than twice as many neurons as survived without exogenous growth factors, while NGF supported five times as many neurons as survived without adding growth factors (Figure 8, lower panels).

The effects of GDNF on neuronal survival were highly selective (Figure 8). Specifically, the addition of GDNF resulted in a fivefold increase in the number of Ret-IR neurons surviving (data not shown) and a 20-fold increase in the number of IB4-positive neurons, compared to cultures grown for 2 weeks without growth factors (Figure 8, lower panels). In contrast, GDNF did not significantly increase the number of surviving TrkA-IR neurons. Counts of total CGRP-IR neurons in cultures with GDNF revealed about 1.5 times more neurons compared to control cultures, demonstrating that GDNF had only a minor effect on this population (data not shown).

Dissociated sensory neurons cultured in the presence of NGF yielded a very different pattern of survival. More than twice as many neurons survived in the presence of NGF compared to GDNF, consistent with the fact that 80% of DRG neurons express TrkA in newborn mice, while only 45% of DRG neurons express Ret. As expected, survival of TrkA neurons was increased severalfold by the presence of NGF (Figure 8, lower panels),

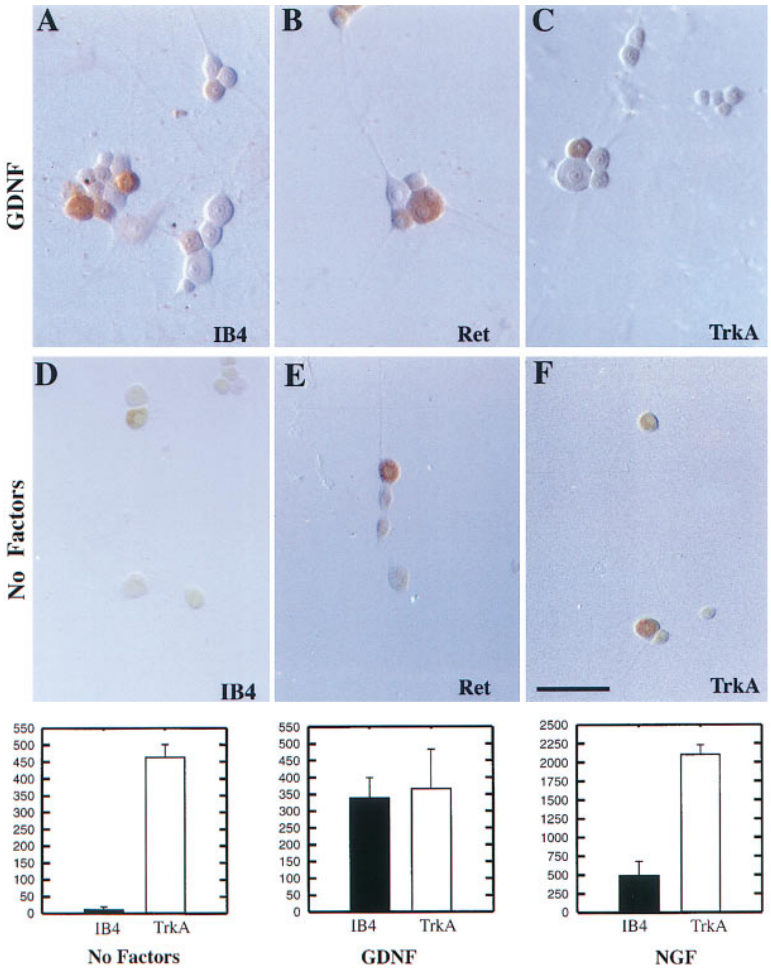

Figure 8. GDNF Selectively Supports the Survival of IB4 and Ret-Positive Neurons In Vitro

Photomicrographs illustrating examples of neurons cultured for 14 days either in GDNF (A–C) or without growth factors (D–F) and immunohistochemically stained for IB4 ([A] and [D]), Ret ([B] and [E]), or TrkA ([C] and [F]). Positive cells are clearly demarcated by the brown DAB reaction product. A low number of neurons survive in the absence of any growth factors. Few of these neurons are IB4-positive (D), and the majority are TrkA-IR (F). GDNF supports the survival of substantial numbers of Ret and IB4 neurons ([A] and [B]). The relatively low number of TrkA-IR neurons surviving in the GDNF condition (C) was similar to that seen in the absence of growth factors (F). Scale bars, 50 μm.

The lower panels show graphs of the mean numbers of surviving neurons stained for IB4 or TrkA that survive in vitro for 14 days in the presence of NGF, GDNF, or in the absence of factors (no factors).

and survival of CGRP neurons was increased by a similar amount (data not shown). NGF also supported substantial numbers of Ret and IB4-positive neurons, consistent with prior observations that all IB4 neurons express TrkA at PN1 (Bennett et al., 1996; Molliver and Snider, 1997).

Discussion

We have shown that the components of the GDNF receptor, Ret and GDNFR-α, are expressed at high levels in the embryonic and adult DRG in patterns that are distinct from that of any of the neurotrophin receptors. Ret is expressed by subsets of large and small sensory neurons that together comprise roughly 60% of neurons in the adult DRG. These include the IB4-binding population of non-CGRP expressing small cells, which we show can be supported by GDNF in vitro. Importantly, the neurons that bind the lectin IB4 undergo a shift in receptor tyrosine kinase expression from TrkA to Ret in early postnatal life, suggesting a switch from NGF to GDNF responsiveness during development.

IB4-Binding Sensory Neurons Express Ret and Respond to GDNF

Interest in the trophic factor regulation of primary nociceptive neurons has been sparked by the remarkable observations that NGF lowers pain thresholds in both rodents and humans and regulates the development and maintenance of inflammation in several experimental paradigms (Lewin et al., 1993; Petty et al., 1994; McMahon et al., 1995; Woolf et al., 1996). However, not all classes of nociceptors appear to be regulated by NGF. Although the great majority of small DRG neurons with unmyelinated axons respond to nociceptive stimuli in rodents and primates (reviewed by Light, 1992), roughly 50% of these neurons do not express the NGF receptor TrkA in adulthood (Averill et al., 1995; Molliver et al., 1995). These non-TrkA-expressing unmyelinated DRG neurons, which lack substance P and CGRP, have been extensively characterized both morphologically and biochemically (see Lawson, 1992 for a review of the older literature; Averill et al., 1995; Molliver et al., 1995). A particularly useful marker has been the lectin IB4, which exclusively binds neurons with unmyelinated axons and which exhibits little or no binding to CGRP and substance P-expressing cells (Silverman and Kruger, 1990; Averill et al., 1995). Neurons binding IB4 have a dense projection to the inner aspect of lamina II of the dorsal horn, in contrast to TrkA- and CGRP-expressing neurons, which terminate in lamina I and the outer aspect of lamina II (Molliver et al., 1995).

Although the specific functional properties of these

IB4-binding sensory neurons have not been directly examined, the majority of neurons with unmyelinated axons in rodents and primates can be assigned to one of several classes of nociceptors (Lynn and Carpenter, 1982; Fleischer et al., 1983; reviewed by Light, 1992; Lewin and Mendell, 1993). Furthermore, both CGRP-positive and IB4-positive neurons are sensitive to the neurotoxic effects of capsaicin, a noxious compound preferentially selective for polymodal nociceptors (Lawson, 1987; Jansco and Lawson, 1988; Pini et al., 1990). However, the lack of expression of CGRP and substance P by the IB4 population, combined with their presumed inability to respond to NGF, has led to the suggestion that IB4-binding neurons are not involved in inflammation (Hunt and Rossi, 1985; Bennett et al., 1996; Molliver and Snider, 1997). In support of this notion, the distribution of axons containing CGRP in the tissues examined (cornea and testes) is different than that of IB4-labeled axons; CGRP-positive axons are associated with vascular structures, consistent with the putative role of this peptide in inflammation, whereas IB4-positive axons are localized diffusely throughout the tissue (Silverman and Kruger, 1988). Interestingly, IB4-binding neurons, but not peptidergic neurons, selectively express the purinergic receptor $P2X_3$, thought to mediate the pain-inducing actions of ATP (Vulchanova et al., 1996, Soc. Neurosci., abstract). Thus, the IB4-positive population may be responsible for the conduction of chemical nociceptive signals generated by peripheral ATP.

It is likely that the identification of selective regulatory factors for IB4-binding neurons will provide tools for the investigation of functional properties of these neurons. Several observations that we report here suggest that IB4 neurons are regulated by GDNF and possibly neurturin. We have shown that mRNA and protein for the signaling component of the GDNF receptor, Ret, are expressed by roughly half of DRG neurons with small cell bodies. Furthermore, colocalization studies showed that $<5\%$ of the Ret population expressed TrkA mRNA, while essentially the entire IB4-positive population displayed Ret-IR. Finally, intense Ret immunostaining was observed in the inner part of lamina II, the dorsal horn target field of the IB4-binding population. Ret is thus the first growth factor receptor shown to be selectively expressed by IB4-binding neurons.

That IB4-binding neurons express functionally active GDNF receptors is suggested by our demonstration of coexpression of GDNFR-α by 89% of Ret-expressing cells and the retrograde transport of ^{125}I-GDNF by adult Ret-expressing DRG neurons. Whether non-GDNFR-α expressing Ret cells express a second GPI-linked molecule with proposed selectivity for neurturin remains to be determined. Recently published analyses of the distribution of this second molecule suggest that many Ret-expressing neurons in DRG may express both GPI-linked binding components (Baloh et al., 1997; Klein et al., 1997). We have also shown that GDNF powerfully and selectively regulates the survival of postnatal IB4-binding neurons in vitro, an ability that should prove useful in further characterizing properties of the IB4 population. Finally, intrathecal administration of GDNF largely prevents the marked decrease in dorsal horn IB4 staining that follows axotomy, with limited effect on the reduction of dorsal horn CGRP (Bennett et al., submitted). This latter observation demonstrates that GDNF regulates properties of nonpeptidergic nociceptors in mature animals.

It should be emphasized that GDNF and NGF are not the only molecules broadly classified as growth factors that might regulate nociceptors. It has previously been shown that the IGF-1 receptor, IGFR, is extensively colocalized with TrkA in the DRG (Reinhardt et al., 1994). Furthermore, at least one cytokine, LIF, is retrogradely transported by a subset of small neurons (Thompson et al., 1996, Soc. Neurosci., abstract). Finally, c-kit, the receptor for stem cell factor, is expressed by a subpopulation of DRG neurons that overlaps with the NGF-responsive population (Hirata et al., 1995). What aspects of nociceptive functions might be regulated by any of these growth factors remains unknown but should be a profitable area for future investigation.

Developmental Regulation of Ret and TrkA Expression

The profound regulation of DRG development by members of the neurotrophin family of molecules is well documented. We now know that throughout late embryonic development (E13–PN1), survival of virtually all nociceptors is regulated by NGF, whereas the survival of virtually all proprioceptors is regulated by NT-3. The role of neurotrophins in regulating the survival of cutaneous low threshold mechanoreceptors during embryonic life, however, remains unclear. Abundant projections to the deep dorsal horn, the central terminal field of these neurons, are present at PN0 in mice with null mutations in each of the Trk loci (Silos-Santiago et al., 1997). In the postnatal period, it is clear that survival of Merkel cell afferents as well as some D-hair afferents is regulated by NT-3, although these afferents appear to be present at PN0 in NT-$3^{-/-}$ mice (Airaksinen et al., 1996; see also Albers et al., 1996). In contrast, BDNF regulates physiological properties of slowly adapting cutaneous low threshold mechanoreceptors, but not their survival (Carroll et al., 1994, Soc. Neurosci., abstract).

The developmental pattern of Ret expression is highly interesting in light of this unresolved issue of cutaneous low threshold mechanoreceptor growth factor dependence. Ret is expressed in DRGs at both the message and protein level at early developmental stages (Pachnis et al., 1993). However, between E11.5 and E15, Ret-IR was seen in only 12% of DRG neurons, and expression was confined to DRG neurons with large cross-sectional areas. Notably, there was little colocalization of Ret with parvalbumin at later developmental stages. Thus, assuming that there is no change in the phenotype of these large Ret-expressing neurons, we hypothesize that many early Ret-expressing cells are cutaneous low-threshold mechanoreceptors. The demonstration of Ret-IR axons with characteristic patterns of projection into laminae III and IV at E17 is consistent with this interpretation. Furthermore, many peripheral Ret-IR axons were closely associated with developing hair follicles in the dermis. Conflicting evidence related to DRG neuron survival in the GDNF knockout leaves unresolved at present the appealing hypothesis that GDNF regulates survival of a

subpopulation of cutaneous low threshold mechanoreceptors during embryonic development (Moore et al., 1996; Sanchez et al., 1996).

The developmental aspects of Ret expression by small DRG neurons are of particular interest. Small neurons did not express Ret until after E15, and the adult pattern did not appear until PN7.5. In contrast, TrkA is expressed by all small DRG neurons between E13 and PN1 and is then selectively downregulated in the IB4-binding population between PN1 and PN21 (Bennett et al., 1996; Molliver and Snider, 1997). Thus, Ret is upregulated in late embryonic and early postnatal life in precisely the population of sensory neurons that downregulates TrkA shortly thereafter. As yet, there are no clues as to the nature of the signals that regulate this phenomenon (Molliver and Snider, 1997). It can be expected that this switch in receptor tyrosine kinase expression from TrkA to Ret is paralleled by developmental changes in expression of GDNF and/or neurturin in tissues innervated by Ret-expressing neurons. Preliminary results indicate that neurturin is expressed at high levels in the skin during late embryonic and postnatal development (Judy Golden and E. M. Johnson, Jr., personal communication). As yet, only imprecise information exists as to GDNF localization in skin (see Wright and Snider, 1995). The recently reported introduction of a *lacZ* reporter into the GDNF locus should provide a means of rapidly localizing putative sites of GDNF synthesis during development (Sanchez et al., 1996).

The developmental increase in the percentage of Ret-expressing neurons in the DRG explains the previously reported increase in efficacy of GDNF as a DRG neuron survival factor with increasing age. Thus, Matheson et al. (1996) found in rat that GDNF supported the survival of $<$10% of sensory neurons at embryonic stages, whereas GDNF supported $\sim$40% as many neurons as NGF at PN0 (see also Buj-Bello et al., 1995; Kotzbauer et al., 1996). Our in vitro data are in close agreement with this latter figure reported in rat and are consistent with the fact that only 45% of DRG neurons express Ret at PN 0.5, whereas 80% of DRG neurons express TrkA at this age. The IB4 population appeared to be especially dependent on growth factor support, as essentially none of these neurons was present in cultures that lacked growth factors. This finding is compatible with the selective vulnerability of this population to axotomy in the neonatal period (see below). GDNF supported the survival of many IB4 neurons while having virtually no effect on the survival of the TrkA population. Apparently, IB4 neurons in the presence of GDNF appropriately downregulate TrkA over 2 weeks in vitro as they do in vivo. NGF also supported substantial numbers of IB4 neurons, which is not surprising, as essentially all of these neurons express TrkA at PN1 (Bennett et al., 1996; Molliver and Snider, 1997).

The developmental profile of Ret and TrkA expression also explains the surprising, recently published finding that virtually all DRG neurons normally lost after neonatal axotomy can be saved by either NGF or GDNF (Matheson et al., 1997). While the identity of the roughly 50% of DRG neurons that die after neonatal sciatic nerve transection has not been determined, in the trigeminal ganglion it has been demonstrated that IB4-binding neurons are selectively vulnerable to neonatal axotomy (White et al., 1990). Our findings suggest that most IB4 neurons coexpress Ret and TrkA at birth. Thus, it is reasonable to conclude that these neurons might be rescued by either NGF or GDNF in the perinatal period, accounting for the roughly equivalent effects of these factors in the neonatal axotomy paradigm (Matheson et al., 1997). At present, it is unclear whether IB4-binding DRG neurons normally require GDNF/Ret signaling for survival rather than for regulation of other properties. Data on this point is lacking, as it is has not been possible to study the fate of IB4 neurons in the postnatal period of GDNF and Ret null mutant mice due to early lethality of the animals.

Conclusion

We have shown that both components of the GDNF receptor are expressed at high levels in the roughly 50% of unmyelinated DRG neurons that express no neurotrophin receptor, and that GDNF selectively supports the survival of these neurons in vitro. These neurons have a distinct projection to lamina IIi of the superficial dorsal horn. We therefore conclude that GDNF is an important regulator of a group of DRG neurons that may contribute to nociceptive processing.

Experimental Procedures

Animals

Pregnant CF-1 mice (Charles River) were deeply anesthetized with sodium pentobarbital (64.8 mg/ml at a dose of 0.4 ml per 100 g body weight), and embryos of different developmental stages were frozen on dry ice for in situ hybridization (at least n = 4 for each age). Late embryonic and neonate pups were overdosed with methoxyflurane and perfused with 3% paraformaldehye/15% picric for protein localization. The fourth and fifth lumbar ganglia were identified by following the sciatic nerve; lumbar DRG and spinal cord were removed, infiltrated with 30% sucrose, and frozen on dry ice. Adult mice were overdosed with sodium pentobarbital, and lumbar DRG and spinal cord were processed identically to neonate tissue. Serial transverse sections (14 μm) were cut on a cryostat, thaw-mounted onto SuperFrost Plus slides (Fisher), and stored at -20°C until used. Some sections were cut at 30 μm to more effectively visualize central projections.

Cloning of *c-Ret* and GDNFR-α Probes for In Situ Hybridization

A 396 bp cDNA fragment corresponding to a region of the extracellular domain (bps 211–607) of mouse *c-Ret* was cloned by RT-PCR using total RNA from E12.5 mouse embryos. RNA was extracted using RNAzol (Tel-Test), and 2 mg of total RNA was reverse-transcribed (SuperScript II, Gibco). PCR was performed in a 50 ml reaction, containing 1$\times$ PCR buffer, 1.5 mM $MgCl_2$, 150 μM dNTP, 0.3 μM of each primer, and 1.25 unit Taq-polymerase (Gibco), in the following conditions: 94°C for 1 min, 64°C for 1 min, and 72°C for 1 min for 35 cycles, followed by a 7 min extension at 72°C. The following 5′ and 3′ primers were used: 5′-TCACACTGATGTTGGGACAA AGG-3′ and 5′-CATCTCTATGGCGTCTACCGTACAC-3′. Sequencing of clones was performed on an Applied Biosystems 373 DNA sequencer using Taq DyeDeoxy Terminator cycle sequencing kits (Applied Biosystems).

Several cDNA fragments from both the rat and mouse GDNFR-α receptor were cloned by RT-PCR using total RNA from adult midbrain, brainstem, liver, and kidney from the rat and heart and liver from the mouse. Four different sets of primers (R1/R3 = 595 bp, R1/R4 = 770 bp, R2/R4 = 527 bp, R7/R8 = 102 bp) matching the published rat GDNFR-α sequence (Jing et al., 1996) were used. Primer sequences were as follows: R1, 5′-GGCATGAAGAAAGAGAA GAATTG-3′; R2, 5′-GCCTACATCACCCCCTGCACCAC-3′; R3, 5′-GCAGATGTAATTGGTCTTGCAGG-3′; R4, 5′-CCATTGCCAAAGGC

TTGAATTGC-3′; R7, 5′-TATGAGCCGGTTAACAGCAGG-3′; and R8, 5′-GCAGTTGTTCCCTTTGGAAATG-3′.

PCR products obtained from different tissues were cloned into the *pCR2.1* vector (Invitrogen). Sequencing of independent clones derived from different primer sets revealed rat and mouse clones identical to the published GDNFR-α sequence. Surprisingly, however several rat and mouse clones had a 15 bp in-frame deletion corresponding to nucleotides 719–733 of the GDNFR-α sequence. The significance of the GDNFR-α deletion is not clear and will require further analysis.

In Situ Hybridization

Riboprobes for in situ hybridization were synthesized from the following cDNA fragments: a 396 bp cDNA encoding a portion of the Ret extracellular domain, a 350 bp cDNA encoding mouse parvalbumin, a 464 bp fragment encoding a portion of the extracellular domain of mouse TrkA (*pDM97*), a 577 bp fragment (*pJDM837*) encoding a portion of the extracellular domain of *trkC* receptor, and a 595 bp fragment encoding a portion of the mouse GDNFR-α. Riboprobes were synthesized from linearized plasmids in the presence of 3.5 mM digoxigenin-11-UTP (Boehringer Mannheim) or 90 μCi [^{33}P]UTP (Amersham) using either T7, T3, or SP6 RNA polymerases.

Briefly, frozen slides were thawed and prehybridized following previous protocols (Wright and Snider, 1995). Sections were hybridized with single [^{33}P]UTP-labeled riboprobes (1×10^6 cpm/slide) or in combination with digoxigenin-labeled riboprobes (500 ng probe/slide) diluted in hybridization buffer. Hybridization mixture (60 μl) was applied to each slide and hybridized overnight at 60°C. Posthybridization washes consisted of sequential rinses in 37°C warmed SSC and RNase-treated for 30 min at 45°C (RNase A, 40 μg/ml). Isotopic-labeled sections were rinsed in distilled H_2O and air dried. Sections hybridized with colorimetric probes were processed to visualize the alkaline phosphatase reaction product according to previous protocols (Wright and Snider, 1995). Slides were then emulsified in Kodak NTB-2 and stored at 4°C for 2 weeks before developing. For controls, sections were incubated with individual sense strand ^{33}P-labeled riboprobes or were pretreated with RNase A (Boehringer Mannheim, 20 μg/ml for 30 min at 37°C) followed by hybridization with individual antisense riboprobes. In each case, control hybridizations resulted in loss of specific hybridization signal.

Analysis

In sections of DRG labeled with isotopic and nonisotopic probes, the percentage of labeled cells was quantified from sections of two L5 DRGs for each of six mice using camera lucida. Neuronal profiles containing nuclei and labeled with nonisotopic probes were drawn under brightfield conditions, and neurons labeled using isotopic probes were drawn under darkfield conditions, at 200× magnification. Positive neurons were identified based on the presence of blue to purple precipitate (nonisotopic riboprobes), whereas isotopically labeled neurons were identified by the presence of silver grains at least five times above background dispersed in a tight circular profile over a neuron. For all photographic figures, 35 mm slides were digitized using a Polaroid SprintScan 35, and Adobe Photoshop 4.0 was used to adjust color and contrast and to arrange figures.

Preparation of ^{125}I-labeled GDNF

GDNF was iodinated to a specific activity ($0.6–2 \times 10^7$ cpm/μg) with Na ^{125}I and lactoperoxidase using the methods of Marchalonis (1969). The reactions were performed at room temperature using the following quantities: 1–5 μg protein in 36 μl of 0.2 M $NaPO_4$ buffer [pH 6.0], 3 μl lactoperoxidase (200–300 μg/ml), 5–10 μl of Na ^{125}I (Amersham, 1 mCi/10×), and 1 μl of a 1:103 dilution of H_2O_2 (30%) in a 0.1 M Na PO_4 buffer (pH 6.0), for a total reaction volume of 50 μl. The reaction was terminated after 15 min with the addition of 150 μl of a 0.1 M Na PO_4 buffer containing 0.42 M NaCl and 0.1 M NaI (pH 7.5). The protein was separated from free counts using a G10 Sephadex column.

Retrograde Transport of ^{125}I-Labeled GDNF

Adult Sprague-Dawley male rats (250–300 g) were anesthetized and the sciatic nerve was exposed and crushed using firm pressure applied to the nerve for 30 s. 1–5 μl (25–100 ng) of radiolabeled protein was injected directly into the nerve at the site of the crush, and 14–16 hr later, animals were perfused transcardially with buffered saline followed by 4% paraformaldehyde. Ipsilateral and contralateral L3–L5 DRGs were removed and counted, and then ipsilateral L4–L5 DRGs were processed for immunohistochemistry (see below). Following antibody treatments, sections were coated with Kodak NTB-2 emulsion and exposed for 4–5 weeks at 4°C before developing. In three experiments, coinjection of 100-fold excess unlabeled GDNF was performed and found to reduce radioactive labeling of DRG sections to background levels, demonstrating the specificity of ^{125}I-GDNF transport. In all, 14 rats were injected with hot GDNF, and 15 rats were injected with both hot and cold GDNF.

Generation of Anti-Ret Antibodies

Antibodies were raised by immunizing rabbits with the Ret-Fc protein purified from CHO cells (Jing et al., 1996). Rabbits were immunized with an initial injection of 200 μg of Ret-Fc. The antigen was emulsified with complete Freund's adjuvant and injected subcutaneously at multiple sites. At 4 week intervals, the rabbits were boosted with 200 μg of antigen in complete Freund's adjuvant injected at multiple subcutaneous sites. Sera were collected by ear vein bleed 10 days after each injection and titered for anti-Ret-Fc antibodies. Anti-Ret-Fc antibodies from selected high titer antisera were purified by affinity chromatography using an Actigel ALD-Ret-Fc affinity resin as recommended by the manufacturer (Sterogene Bioseparations, Arcadia, CA). The affinity gel was made by overnight coupling of 9 mg of recombinant Ret-Fc to 4 ml Actigel ALD. Unreacted aldehyde groups were deactivated with ethanolamine. About 50 ml of pooled antisera was added to the Ret-Fc affinity gel, and the slurry was gently agitated overnight at 40°C. The serum was drained from the gel and the gel washed with PBS to remove unbound serum proteins. Bound anti-Ret-Fc antibodies were then eluted with ImmunoPure Gentle Ag/Ab Elution Buffer (Pierce Chemical). Eluted antibodies were dialyzed against PBS. These recovered antibodies are a mixture that recognizes the extracellular domain of Ret receptor as well as the Fc portion of human IgG. To remove those antibodies recognizing the Fc portion and recover only those antibodies recognizing Ret, the antibody pool was exposed to an Actigel ALD coupled with human IgG. The IgG affinity gel run-through contained anti-Ret antibodies. This solution was concentrated in a stirred cell ultrafiltration unit (Amicon) to ~1 mg/ml specific anti-Ret antibodies.

Immunocytochemistry

Sections were incubated in blocking buffer consisting of 1.5% normal goat serum, 1% porcine gelatin, and 0.2% Triton X-100 in Superblock buffer (Pierce). The Ret antibody was used at 1 μg/ml; IB4 conjugated to HRP or fluorescein (Sigma) was used at 10 μg/ml; rabbit anti-CGRP (Peninsula) was used at a dilution of 1:1,000; and a rabbit anti-TrkA antibody, kindly provided by Dr. Louis Reichardt, was used at a dilution of 1:10,000. Primary and secondary antibodies were diluted in the same solution, which was diluted 1:1 with Superblock buffer/1.5% normal serum. Sections were incubated in primary antibody overnight, washed in PBS, and placed in secondary antibody incubation for 30 min. For horseradish peroxidase-diaminobenzidine (HRP-DAB) visualization of antibody staining, sections were removed from primary antibody, washed in PBS, and processed with a Vectastain kit (Vector, Burlingame, CA). For double-labeling of IB4 and Ret, CY3 conjugated to donkey anti-rabbit (Jackson Immunoresearch Laboratories, West Grove, PA), at a dilution of 1:200, was used as a fluorescent secondary antibody for visualization of Ret in combination with IB4 directly conjugated to FITC. As a negative control, representative sections were processed without a primary antibody. Preabsorption with 90-fold excess of Ret-Fc (by weight) completely blocked the anti-Ret staining in sections of adult CNS.

For visualization of central projections at E17.5, a biotin-tyramide amplification step was performed to enhance resolution, according to instructions in a "TSA-Indirect" tyramide kit (NEN Life Sciences). Staining was visualized with the HRP-DAB reaction.

Quantitation

In order to obtain an unbiased percentage of adult DRG neurons expressing Ret, Ret-IR neurons were counted using the physical

dissector method as follows (Pakkenberg and Gundersen, 1988; Coggeshall and Lekan, 1996). Six L5 DRGs from four adult (~40 g) female mice were sectioned in their entirety at 10 μm and stained with the Ret antibody. Every twelfth section was analyzed, starting with a randomly chosen section between 1 and 12, and compared to an adjacent section. Each pair of sections was photographed and digitized for analysis. Only neurons with clearly visible nuclei and nucleoli present in the first but not the adjacent "look-up" section were counted. The percentage of Ret-positive neurons was obtained from 674 neurons in six L5 ganglia. Size frequency histograms were then generated from counted neurons.

Percentages and size frequency histograms of TrkA and Ret-expressing neurons in lower lumbar ganglia from embryos were prepared as described in Molliver et al. (1995) and Molliver and Snider (1997). At least three embryos for each receptor at each developmental age were studied. The use of profile counts in these embryos could have resulted in a slight overestimation of the percentage of Ret-expressing cells and a slight underestimation of the percentage of TrkA-expressing cells.

In Vitro Experiments

For each experiment, 5 PN0.5 mouse pups were decapitated, and DRGs were dissected and collected in L15 media. In order to dissociate the neurons, DRGs were incubated in collagenase (Sigma) for 10 min and then in trypsin (Sigma) for 6 min, washed in MEM, and triturated. Dissociated cells were plated in 4-well chamber slides coated with poly-D-lysine and laminin at 10,000 cells per well as determined by hemocytometer. Cells were plated in media containing MEM, 2 mM glutamine, 10% fetal calf serum, and pen/strep solution. To reduce the number of nonneuronal cells, 20 μM FUDR/uridine was added to all media. This treatment, however, did not totally eliminate the nonneuronal cells. In addition, media contained NGF (50 ng/ml), GDNF (Promega; 50 ng/ml), or no trophic factors. This concentration of GDNF was chosen to provide a maximal survival effect for sensory neurons based on dose-response curves from a previous study (Matheson et al., 1997). Media were replaced on the day after plating and every third day. Cells were maintained in culture for 2 weeks and then fixed in the picric acid fixative described above for 30 min and then for 10 min in cold 100% methanol. Cultures were processed for Ret, TrkA, IB4, or CGRP immunocytochemistry visualized with the HRP/DAB reaction as described above, with the exception that the DAB solution contained 12 ng/ml DAB instead of 50 ng/ml. As a negative control, several wells were processed without primary antibody. Slides were dehydrated and coverslipped, positive and negative cells were counted, and total cell counts per well were estimated by counting a lane through the center of each well comprising 20% of the well, based on positioning of the slide with a stage micrometer. The percentage of positive and negative neurons was determined for each marker in each trophic factor condition and compared to average total cell counts per well. Numbers represent the means per well of all experiments. A minimum of six wells were counted for each histochemical marker for each condition. The entire procedure was repeated, for a total of four experiments.

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