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A rapid and dynamic regulation of GDNF-family ligands and receptors correlate with the developmental dependency of cutaneous sensory innervation

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SUMMARY

Glial cell line-derived neurotrophic factor (GDNF) and neurturin (NTN) are members of the transforming growth factor-β family and have been shown to elicit neurotrophic effects upon several classes of neurons including dopaminergic neurons, motoneurons, parasympathetic, sympathetic as well as primary sensory neurons. However, there is little information available on their roles in cutaneous innervation. Herein, we have studied the regulation of gdnf, ntn and the GDNF family receptors and examined their role in the development of facial cutaneous innervation in GDNF mutant mice. A dynamic spatial and temporal regulation of gdnf, ntn and their ligand binding receptors within the follicle-sinus complex correlate with development of distinct subclasses of sensory nerve endings. Furthermore, development of NGF-dependent myelinated mechanoreceptors, i.e. reticular and transverse lanceolate endings also require GDNF during ending formation and maintenance. In addition, ligand and receptor association seems to be intricately linked to a local Schwann cell–axon interaction essential for sensory terminal formation. Our results suggests that functionally specified nerve endings depend on different GDNF family members and that in contrast to neurotrophins, this family of neurotrophic factors may be acting at local sites of terminal Schwann cell-axon growth cone interactions and that they collaborate with neurotrophins by supporting the same populations of neurons but at different times in development.

Key words: GFRα, Ret, Mechanoreceptor, Schwann cell, Trigeminal ganglion, Mouse

INTRODUCTION

Glial cell line-derived neurotrophic factor (GDNF) family ligands are secreted molecules that play fundamental roles during inductive events of organogenesis as well as in cell survival and differentiation in the developing nervous system. Recently, components of the receptor system mediating the effects of GDNF and neurturin (NTN) were discovered. The glycosyl-phosphatidyl-inositol (GPI) membrane-linked receptor subunit, GDNFRα/TrnR1 which will be referred to as the GDNF-family receptor α1 (GFRα1) binds GDNF. The complex GDNF-GFRα1 is required for subsequent binding and activation of the tyrosine kinase Ret receptor (Treanor et al., 1996; Trupp et al., 1996). Two additional receptors TrnR2/NTNR-a/RetL1 (GFRα2) and GFRα3 displaying close to 50% and 32% amino acid homology to GFRα1, respectively, were recently identified and characterized (Baloh et al., 1998a, 1997; Buj-Bello et al., 1997; Jing et al., 1997; Klein et al., 1997; Masure et al., 1998; Naveilhan et al., 1998; Sanicola et al., 1997; Widenfalk et al., 1998; Worby et al., 1998). GDNF and NTN can activate Ret in cultured cell lines by interacting with either GFRα1 or GFRα2 (Creedon et al., 1997). However, when present at low concentrations in a neuronal context, GDNF and NTN display a preference for GFRα1 and GFRα2, respectively (Buj-Bello et al., 1997; Naveilhan et al., 1998). In contrast, the GFRα3-Ret receptor complex is activated only by the newly discovered ligand artemin (Baloh et al., 1998b). However, artemin was also capable of activating the GFRα1-Ret receptor complex. Recently, GFRα4 has been cloned in chicken (Thompson et al., 1998) and was shown to form a functional receptor complex with Ret which could not be activated by either NTN or GDNF (Enokido et al., 1998). A novel member of the TGF-β family of ligands, persephin (Milbrandt et al., 1998), was shown to bind and activate the GFRα4-Ret receptor complex in vitro (Enokido et al., 1998).

The ligand binding receptors GFRα1, GFRα2 and GFRα3 are expressed in distinct and segregated patterns within the trigeminal ganglion, i.e. GFRα2 in ventral and GFRα3 in dorsal neurons whereas GFRα1 is expressed in neurons scattered throughout the ganglion (Naveilhan et al., 1998). Double in situ hybridization has revealed that GFRα2 and GFRα3 are preferentially expressed in different cell

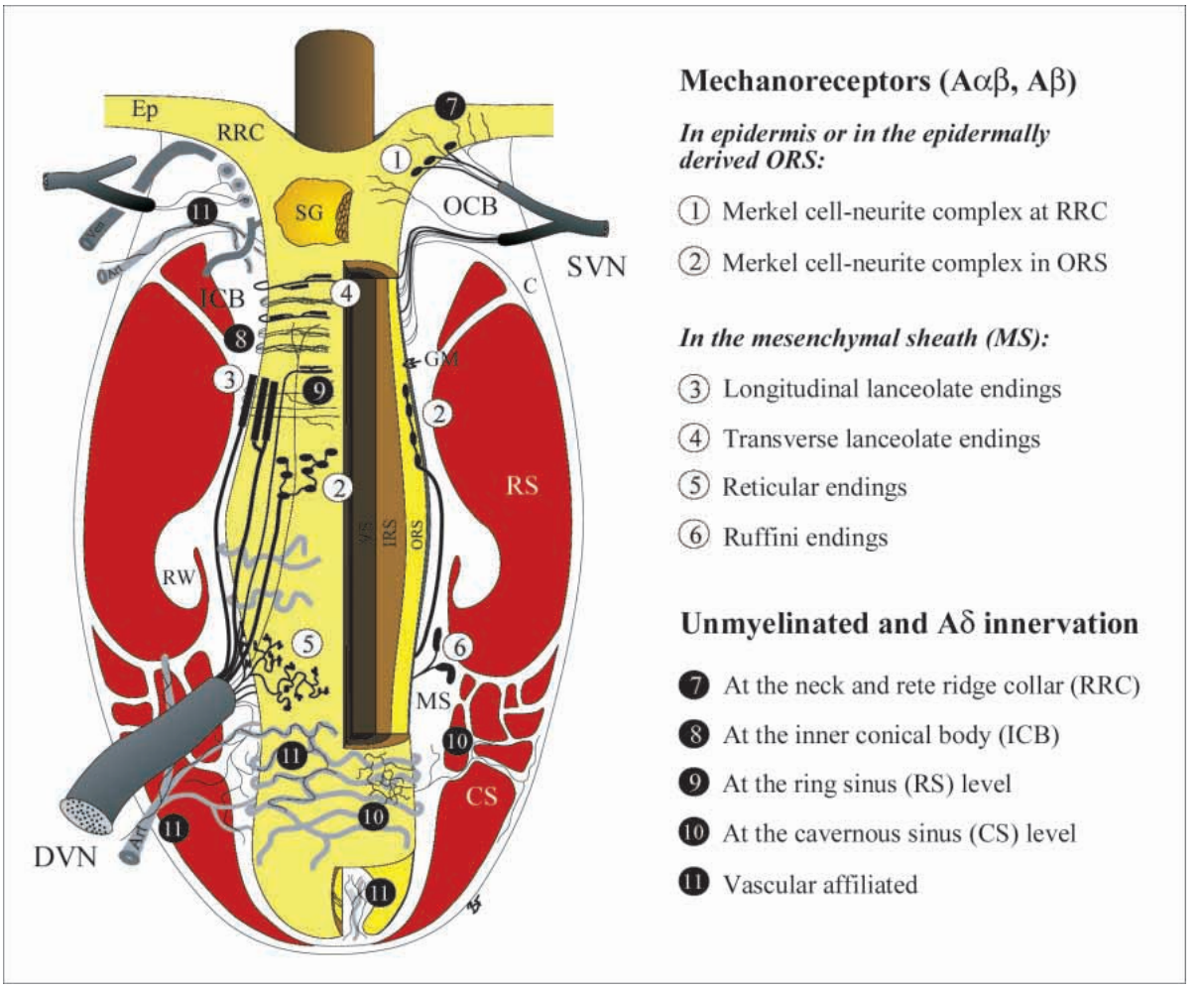

Fig. 1. Schematic drawing illustrating the structure and innervation of the follicle-sinus complex (FSC). The epidermal-derived follicle (light yellow) encased within a dermal-derived vascular sinus (red) is divided into the ring sinus (RS) and the cavernous sinus (CS). The follicle is composed of inner and outer root sheaths surrounded by a thick basement membrane referred to as the glassy membrane (GM). At the mouth of the follicle, the epidermis (Ep) thickens to form a rete ridge collar (RRC). A dense, collagenous capsule (C) encloses the sinus and expands as it converges upon the neck of the follicle to form the outer conical body (OCB) which contains a sebaceous gland (SG). At the base of the FSC, the capsule merges with the follicle which is penetrated by a vascularized dermal papilla (DP). A mesenchymal sheath (MS) composed of loose connective tissue coats the glassy membrane and expands at the neck to form the inner conical body (ICB). A doughnut shaped ringwulst (RW) is suspended from the circumference of the mesenchymal sheath. The innervation supplied by the deep vibrissal nerve (DVN) and the superficial vibrissal nerves (SVNs) is listed to the right, with the corresponding numbers in the figure indicating the location of the different sets of innervation.

populations whereas GFRα1-positive neurons mostly coexpress GFRα2 or GFRα3 (Naveilhan et al., 1998). Buj-Bello et al (1995), showed that GDNF promotes the survival of primary cultures of embryonic chick trigeminal ganglion neurons. Their results further suggest that NGF- as well as BDNF-responsive neurons acquire GDNF-responsiveness with increasing age.

The well-defined whisker follicle-sinus complex (FSC) in rodents receives its innervation from the infraorbital nerve, a maxillary branch from the trigeminal ganglion. A deep vibrissal nerve and several smaller superficial vibrissal nerves give rise to five functional types of myelinated mechanoreceptors as well as a large number of unmyelinated peptidergic/non-peptidergic neuronal networks (Fundin et al., 1994; Rice et al., 1997, 1993). As shown schematically in Fig. 1, the innervation to the FSC is arranged in a very strict and specific pattern that allows detailed studies of neurogenesis and plasticity in the peripheral nervous system. Although most axons have reached the target tissue already at E13 (Davies and Lumsden, 1984), terminal innervation and sensory ending formation spans at least three weeks and occurs orderly for the functionally different sets of sensory neuron during this period.

In order to understand the specific roles of the GDNF family members of neurotrophic factors during development of the peripheral nervous system we have characterized their expression and function within the whisker follicle-sinus complex and found: (1) that a dynamic regulation of gdnf, ntn and their ligand binding receptors within a highly evolved cutaneous target coincides with the development of specific sets of sensory innervation; (2) a dependency of several classes of myelinated NGF-dependent mechanoreceptors on GDNF signaling; (3) an intricate interaction of GDNF-family ligands

and receptors occurs between the arriving axon and the terminal Schwann cells; and (4) a possible role for GFRα3 in regulating the action of NTN.

MATERIALS AND METHODS

Probes

The rat gfrα1 and ret probes used in this assay, which have previously been described in Naveilhan et al. (1997) correspond to nucleotides 862-1160 (Jing et al., 1996) and 1381-1750 (Iwamoto, 1993). The rat gdnf, the mouse gfrα2, gfrα3, and ntn cDNA probes corresponding to nucleotides 380-800 (Lin et al., 1993), 805-1215 (Baloh et al., 1997), 601-910 (Naveilhan et al., 1998) and 613-963 (Kotzbauer et al., 1996), respectively, was prepared as described by Naveilhan et al. (1998).

In situ hybridization procedure

For in situ hybridization, tissues of time-staged mouse embryos, postnatal day 0 (P0), P3, P7, P14 and adult were positioned depending on stage on a metal block as whole embryos or following dissection of the mystacial pad, frozen and sectioned (14 μm) on a Leitz cryostat. All sections were thaw-mounted onto slides pretreated with 3-aminopropyl triethoxysilane (Sigma) and kept frozen until hybridization. A non-radioactive digoxigenin UTP-ribonucleotide in situ hybridization was employed. In brief, the slides were dried at room temperature (RT) for 30-45 minutes before fixation in 4% paraformaldehyde (PFA) for 15 minutes (RT). The slides were then washed twice in PBS for 5 minutes (RT) and once in distilled water for 5 minutes (RT), followed by a deproteinisation step with 0.1 M HCl treatment for 15 minutes (RT). The slides were washed twice in PBS for 5 minutes (RT) followed by 20 minutes incubation with 0.25% acetic anhydride in 0.1 M triethanolamine (RT) and a second wash twice in PBS (RT). The slides were then prehybridized for 4 hours in hybridization buffer (50% formamide, 5× SSC, 1 mg/ml yeast tRNA, 1× Denhardt's solution, 0.1% Tween 20, 0.1% CHAPS, 5 mM EDTA) at 60°C before hybridization with the probe (40 ng/slide in 200 μl hybridization buffer) overnight. The slides were given a quick wash in 5× SSC (standard saline citrate, 60°C), then twice in 2× SSC 15 minutes (60°C and 37°C, respectively), RNase A treatment (0.2 μg/ml in 2× SSC) for 30 minutes (37°C) followed by a 15 minute wash in PBS + 0.1% Triton X-100 (60°C) and PBS + Triton X-100 + 2 mg/ml bovine serum albumin (PBT) for 15 minutes at RT. The slides were then incubated in PBT + 10% heat inactivated goat serum (HIGS) for 5 hours (RT) followed by the addition of the anti-DIG antibody (1:2000; Boehringer Mannheim) diluted in PBT + 10% HIGS and incubation overnight at 4°C. Before addition of the substrate, the slides were washed three times in PBS + Triton X-100, twice for 15 minutes (RT), and once for 30 minutes (RT) followed by a 5 minute wash in alkaline phosphatase buffer (100 mM Tris pH 9.5, 50 mM $MgCl_2$, 100 mM NaCl, 0.1% Tween 20) containing levamisol (5 mM; Sigma,) and a 24-72 hour incubation with substrate, i.e. NBT (nitro blue tetrazolium; Boehringer Mannheim) and BCIP (5-bromo-4-chloro-3-indoyl phosphate; Boehringer Mannheim) 3.5 and 3.0 μl/ml alkaline phosphatase buffer + levamisol, respectively. When the staining was satisfactory, the slides were washed once in PBS and fixed with MEMFA (0.1 M MOPS pH 7.5, 2 mM EGTA, 1 mM $MgSO_4$, and freshly added 3.7% formaldehyde) for 15 minutes and mounted in PBS:glycerol (1:9).

Genotyping of GDNF mutant mice

Heterozygous GDNF mutant mice were bred, and their offspring were collected at birth for the experiments. All of the neonatal mice were genotyped for the wild-type and gdnf mutant alleles by PCR according to Pichel et al. (1996). Adult and P5 heterozygous GDNF mutant mice as well as P0 homozygous GDNF mutant mice were used in this study.

Immunohistochemistry

For optimal immunohistochemistry with anti-PGP 9.5, anti-CGRP and RT97, the mystacial pads were removed and postfixed at 4°C in the perfusion fixative for 1 hour, rinsed in PBS and cryoprotected by overnight infiltration with 20% sucrose in PBS. 14 μm thick sections were cut on a cryostat perpendicular to the skin surface and parallel to the rows of follicles. This resulted in sections oriented approximately along the length of the FSCs. Some specimens were cut parallel to the skin surface resulting in cross sections of the FSCs. The sections were directly mounted onto slides pretreated with 3-aminopropyl triethoxysilane and air-dried. For optimal immunohistochemistry with anti-GFRα1, fresh-frozen sections were cut as described above followed by fixation on slides in 4% PFA for 15 minutes.

Immunofluorescence analyses were performed by the protocol described by Fundin et al. (1997a). Briefly, we used a polyclonal antibody against a pan-neuronal cytoplasmic antigen, protein gene product 9.5 (PGP 9.5) (diluted 1:500, Ultraclone Ltd.), which labels all known neuronal structures in the skin (Rice et al., 1993). A monoclonal antibody, RT97 (1:500; Peninsula, Inc.), against a 200 kDa phosphorylated neurofilament protein was used which generally labels cell bodies, axons and most endings of myelinated neurons (Fundin et al., 1997a; Rice et al., 1997). Sections were also prepared with rabbit anti-GFRα1 antisera (1:100; gift from Carlos Ibanez) or rabbit anti-calcitonin gene-related peptide (CGRP) (1:800, Peninsula Inc.) which is a broadly distributed neuropeptide. To detect polyclonal primary antibodies (i.e. anti-PGP 9.5, anti-CGRP and anti-GFRα1), donkey anti-rabbit secondary antisera conjugated to cyanine-3.18 (Cy3) (1:500; Jackson Immuno Research Laboratories, Inc.) were used. To detect the monoclonal primary antibody, RT97, donkey anti-mouse conjugated to FITC (1:50; Jackson Immuno Research Laboratories, Inc.) was used. The slides were then rinsed in excess PBS and mounted with 0.1% paraphenylene diamine (PPD) in glycerol.

Double labeling with the polyclonal anti-GFRα1 and the monoclonal RT97 primary antibodies was performed by first incubating with one of the desired primary antibodies followed by the appropriate Cy3-conjugated secondary antibody, and second, incubating with the other primary antibody followed by its appropriate FITC-labeled secondary antibody.

Analysis

Sections were viewed with a Nikon Microphot FXA equipped for TRITC and FITC epifluorescence. The images were captured and processed by using the IP-lab spectrum software (Signal Analytics, Vienna, VA). All five rows (A-E) of vibrissal FSCs on each side were included as well as the larger α, β, δ and γ FSCs that straddle the caudal ends of the rows.

For confocal images, a Zeiss LSM 510 confocal microscope was used. Briefly, the 543 nm line of an NeonHelium ion laser was used as the excitation light, and the Cy3 fluorescence emission light was collected through a dichroic mirror and a long pass filter (LP 560 nm). The images were recorded with either ×63/1.40 oil DIC-immersion planapochromate, ×25/0.80 Imm. Korr. DIC plan neofluar or ×10/0.45 planapochromate objective. The lateral pixel spacing was set to 1.80 μm, 0.51 μm or 0.14 μm and the z-axis stepping size to 2.80 μm, 0.80 μm or 0.45 μm for Fig. 3G,H and 9C,D, respectively. Series of 22 (Fig. 3G,H) or 61 (Fig. 9C,D) consecutive optical sections were recorded from each specimen. Stereo pairs were made with a total of 12° angle between the image stacks.

RESULTS

A rapid and dynamic expression of GDNF-family ligands and receptors in the trigeminal system

Expression of GDNF-family receptors in the trigeminal ganglion

In the trigeminal ganglion at E13, large proportions of diffusely distributed neurons expressed ret, gfrα3 and gfrα1 (Fig.

2A,B,D) whereas only a small proportion expressed gfrα2 (Fig. 2C). At E18, gfrα1 and gfrα3 was down regulated in many trigeminal neurons, whereas gfrα2 and ret expression appeared similar to that observed at E13 (Fig. 2E-H). While gfrα2 expressing neurons were preferentially located ventrally and gfrα3 dorsally, gfrα1 and ret neurons were scattered throughout the ganglion. This arrangement was even more evident at birth (Naveilhan et al., 1998).

Regulation during early axonal target tissue invasion

At embryonic day 11, pioneering axons from the trigeminal ganglion reached the developing mystacial pad (arrows in Fig. 3G,H). As shown in Fig. 3A, expression of gdnf mRNA was evident in the epidermis in a discrete punctuated pattern. In addition, ntn and gfrα3 were both expressed throughout the entire epidermis and mesenchyme in the developing whisker pad (Fig. 3B,F). In contrast, only weak expressions of ret, gfrα1 and gfrα2 were evident in mesenchyme at this stage of development (Fig. 3C-E). At E13, when most axons have reached the target tissue (Davies and Lumsden, 1984) expression of ntn and gfrα3 was down regulated in epidermis and mesenchyme and was restricted to the epidermal-derived whisker follicles (Fig. 4B,F), whereas gfrα2 was expressed in the immediately surrounding mesenchyme (Fig. 4E). As observed with anti-PGP 9.5, the surrounding mesenchyme accommodated several axons none of which have yet penetrated the basement membrane to innervate the developing Merkel cells in the epidermal-derived whisker follicle (Fig. 4G,H; see also Fig. 1).

Regulation of ntn during early Merkel cell-neurite complex formation

At E16, Merkel cells located in the outer root sheath and the rete ridge collar of the whisker follicles established their connections with the invading Merkel axons (Fig. 5E,F; see also Fig. 1). The formation of Merkel cell-neurite complexes, however, continued well into the first postnatal week in the mouse. Similar to that observed at E13, expression of ntn was evident in the outer root sheath of the follicle at E16 (Fig. 5A,B), while gfrα2 was expressed in the surrounding mesenchymal sheath (Fig. 5C,D). In contrast to E13, however, the expression of ntn was down regulated in much of the sheath and restricted only to the level of the developing Merkel innervation (arrows in Fig. 5B) at the ring sinus. Moreover, the gfrα3 expression, which accompanied the ntn expression at E13 (see Fig. 3F), was absent at E16. The mesenchyme at the level of upper cavernous sinus, through which the arriving Merkel axons pass, expressed both ret, gfrα1 and gfrα2 (not shown).

Regulation of gdnf during longitudinal lanceolate innervation

In addition to the continuous formation of Merkel cell-neurite complexes in the outer root sheath at the level of ring sinus, longitudinal lanceolate endings were formed in the surrounding mesenchymal sheath at E18 (Fig. 6F,G; see also Fig. 1). This was accompanied by expression of gdnf in the outer root sheath (arrow in Fig. 6D) and gfrα1 in the surrounding mesenchymal sheath (arrow in Fig. 6E). The expression of ntn and gfrα2, which was evident at E16 (Fig. 5A-D) in this location was absent by E18 (Fig. 6A,C). In contrast, ntn and gfrα3 were upregulated in the outer root sheath at the upper cavernous level (arrowheads in Fig. 6A,B), which at this stage received reticular axons (arrowhead in Fig. 6F, see also 6G).

Regulation during Ruffini, reticular and transverse lanceolate ending formation

In 3- and 7-day postnatal mice, gdnf, gfrα1, gfrα2 and gfrα3 were all expressed in the inner conical body (ICB) at the time

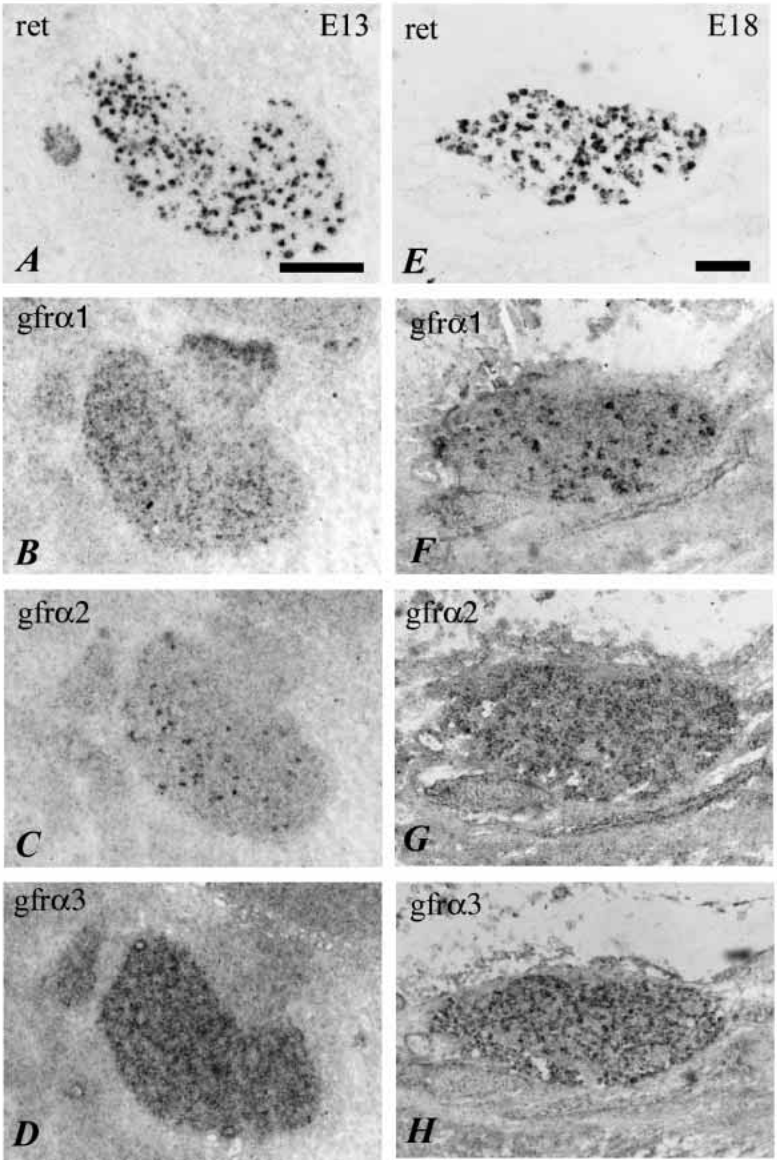

Fig. 2. Expression of the GDNF-family receptors in the mouse trigeminal ganglia at E13 (A-D) and E18 (E-H). While ret expression was equally evident at E13 (A) and E18 (E), gfrα1 was weakly expressed at E13 (B), to be more evident at E18 (F). Although gfrα2 was expressed in a subpopulation of trigeminal neurons at E13 (C), more cells expressed gfrα2 at E18 (G). In contrast, the dense expression of gfrα3 mRNA at E13 (D) was somewhat reduced at E18 (H). Scale bar 100 μm (in A applies to A-D; in E applies to E-H).

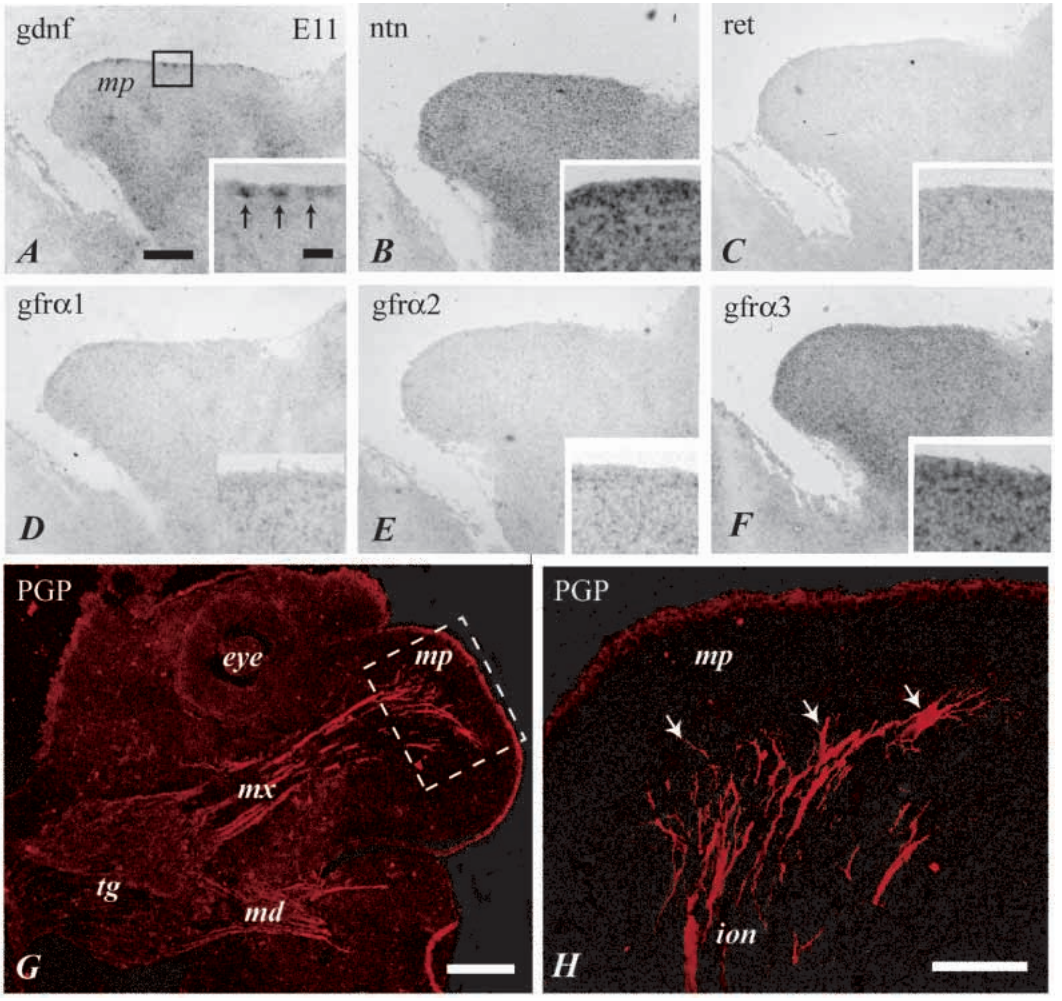

Fig. 3. Expression of the GDNF-family ligands and receptors during early morphogenesis of the whisker follicles. (A-E) Expression at E11 in the developing mystacial pad (mp). Higher magnification of the developing epidermis is shown as inserts. **A**. In the developing mystacial pad, gdnf mRNA was expressed only in the epidermis (arrows) at the location of the forming whisker follicles. (B) ntn mRNA was densely expressed throughout the epidermis as well as in the dermis. (C-E) In contrast, ret (C), gfrα1 (D) and gfrα2 (E) were not expressed at this stage of development. (F) The only receptor expressed at this age was the gfrα3, which had a similar distribution as ntn (B). (G,H) The infraorbital nerve (ion), a division of the maxillary branch (mx) of the trigeminal ganglion (tg), had, at E11, reached the developing mystacial pad (mp). Mandibular branch (md). Scale bar in A for A-F 250 μm and insert 50 μm; in G 200 μm; in F 100 μm.

for the terminal invasion of this region (Fig. 7A,C-F). The innervation to the ICB consisted mainly of unmyelinated or lightly myelinated axons as well as a Aβ-caliber myelinated mechanoreceptor, i.e. the transverse lanceolate ending (open arrowhead in Fig. 7A,H; see also Fig. 1). During the first postnatal week, at the level of reticular ending termination, i.e. the upper cavernous sinus (arrows in Fig. 7A,G; see also Fig. 1), a dense expression of gdnf in the outer root sheath was accompanied by expression of gfrα1 in the surrounding mesenchymal sheath (arrows in Fig. 7D,F,G). In addition, ntn, gfrα2 and gfrα3 were evident in the outer root sheath and to a lesser degree in the surrounding mesenchymal sheath in which the Ruffini endings terminate (arrows in Fig. 7B,C,E).

In 2-week old mice the expression was down regulated and only weak expression of either receptor or ligand was evident in the whisker follicle (not shown), but, in a similar pattern to that observed at E7. In adult whisker follicle GDNF-family ligands and receptors were further down regulated to levels below detection and only cells in the mesenchyme at the level of upper cavernous sinus expressed either gdnf, ntn, ret, gfrα1 or gfrα3 (not shown). In addition to the areas of the FSC that receive neuronal innervation, the growth zone of the whisker follicle, i.e. the root sheath adjacent to the hair inductive dermal papillae (see Fig. 1), showed dense expression of all studied ligands and receptors from embryonic day 16 to postnatal day 14 (e.g. gdnf, asterisk in Fig. 6D). In the adult animal, only weak expression was evident (not shown).

A selective loss of myelinated NGF dependent sensory nerve endings in GDNF mutant mice

In adult GDNF$^{+/-}$ mice there was a dramatic decrease in the Aβ-caliber myelinated NGF-dependent subclasses of sensory nerve endings, i.e. reticular endings and transverse lanceolate endings, as revealed by anti-PGP 9.5 (Fig. 8A-F). In the ICB, the RT97-IR myelinated transverse lanceolate endings were substantially reduced (arrows in Fig. 8A,B), whereas the large proportion of NGF-dependent unmyelinated RT97-negative axons at the same target, including a subpopulation of CGRP-IR profiles, were not affected (arrows in Fig. 8C,D).

The other set affected in adult GDNF$^{+/-}$ mice was the myelinated Aβ-caliber mechanoreceptor, referred to as reticular endings (arrows in Fig. 8E,F). This set is located in the mesenchymal sheath at the upper cavernous sinus level in close association with the basal lamina of the whisker follicle (Fig. 1). The few surviving reticular endings in the adult GDNF$^{+/-}$ mice contained only a few branches unlike wild-type mice where these endings were elaborate with many branches (arrows in Fig. 8F; compare arrows in Fig. 8E). The BDNF-dependent Ruffini endings at the same level, however, seemed

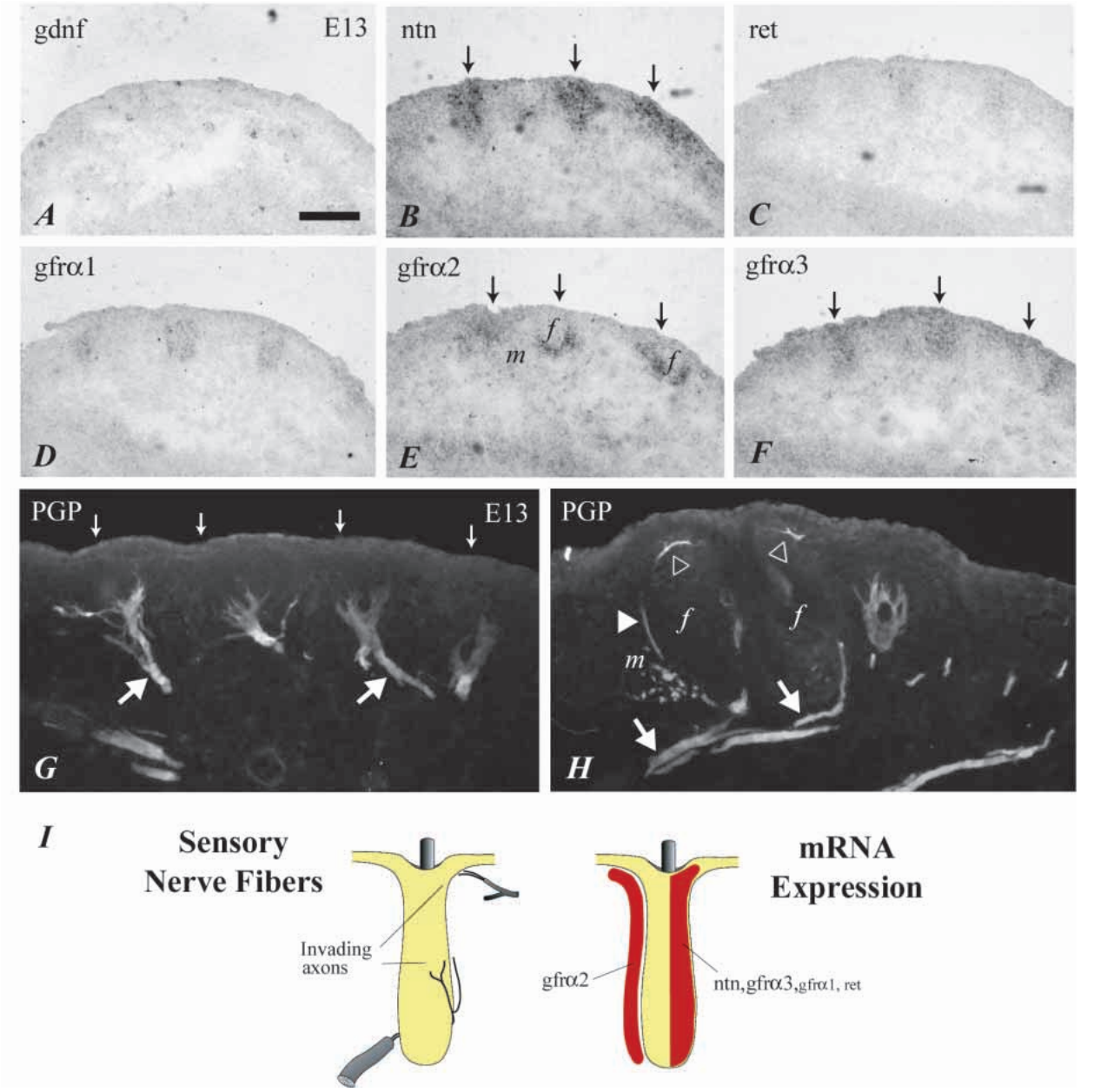

Fig. 4. Expression of the GDNF-family ligands and receptors during early axonal target tissue invasion. Small arrows indicate the location of the developing whisker follicles (f) surrounded by the mesenchyme (m). (A-F) mRNA expression (arrows) of gdnf (A), ntn (B), ret (C), gfrα1 (D), gfrα2 (E) and gfrα3 (F) in the mystacial pad at E13. (G,H) The axonal invasion as revealed with anti-PGP 9.5 at E13. Large arrows indicate the individual deep vibrissal nerves. (G) The invading axons are located in the immediate mesenchyme surrounding the developing whisker follicle (small arrows). (H) Image of larger caudal whisker follicles revealing the axons to the level of ring sinus (arrowhead) as well as to the rete ridge collar (outlined arrowheads). (I) Schematic drawing summarizing the stage of innervation and mRNA expression at E13. Smaller letters indicates weaker mRNA expression. Scale bar, 100 μm.

normal in both number and morphology in all GDNF mutant mice studied (outlined arrowheads in Fig. 8E,F).

Although reticular endings were detrimentally affected by the reduction of GDNF in adult GDNF$^{+/-}$ mice, their axons were present and appeared normal in neonatal GDNF$^{-/-}$ mice (arrows in Fig. 8G,H). However, at this stage reticular endings were immature with only a few branches in both wild-type and mutant. Similarly, in 5-day old postnatal GDNF$^{+/-}$ mice the transverse lanceolate axons were present and comparable with those of wild-type mice (arrows in Fig. 8I-L), suggesting that these sets of NGF-dependent neurons switch to GDNF-dependency postnatally.

Receptor expression in terminal Schwann cells and distal axons

As revealed by in situ hybridization and immunohistochemistry (Fig. 9), gfrα1 mRNA was expressed in the terminal Schwann cells (arrows in Fig. 9E) and the

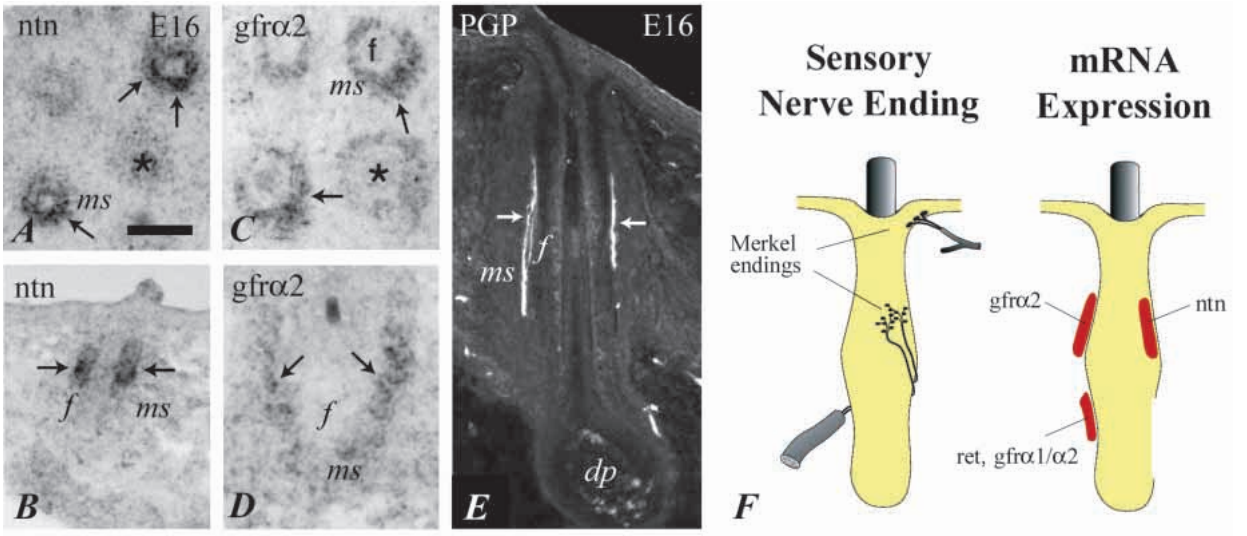

Fig. 5. Specific expression of ntn and gfrα2 during early Merkel innervation. (A-D) Expression (arrows) of ntn (A,B) and gfrα2 (C,D) in the developing whisker follicles at E16. A and C are cross sections of the whisker follicles (asterisks) while B and D are cut perpendicular to the follicles (f). The expression of ntn were strictly located in the outer root sheath at the level of developing Merkel cell-neurite complexes (A,B, see also E and F). Complementary to the ntn expression, gfrα2 was expressed in the mesenchymal sheath (ms) that holds the axons (C,D, see also E). (E) The developing Merkel innervation (arrows) as revealed by anti-PGP 9.5. Dermal papilla (dp). (F) Schematic drawing summarizing the innervation and expression at E16. In addition to the ntn and gfrα2 mRNA observed at the level of ring sinus, expressions of ret, gfrα1 and gfrα2 were evident in the mesenchyme below the target of Merkel endings. Scale bar, 100 μm.

protein was evident preferentially at their apical side and throughout their entire long processes (arrowheads in Fig. 9A,C,D). Double-staining with anti-GFRα1 and RT97 further revealed that the arriving axons (large arrows in Fig. 9A) intimately followed the Schwann cell processes to finally terminate at their correct targets.

Interestingly, the reticular endings (outlined large arrow in Fig. 9A indicate their axons) and the transverse lanceolate endings (not shown) did not show any detectable levels of GFRα1 protein although they were detrimentally affected in the GDNF mutant. Thus, terminal Schwann cells might be a source of GFRα1 utilized by the terminal axons. In addition, terminal Schwann cells also expressed GFRα2 and GFRα3 mRNA, which further suggests a generality spanning all GDNF-family receptors in regulating terminal innervation. Similarly, the longitudinal lanceolate axon/terminal (small arrowheads in Fig. 9B) as well as the Merkel axon (large arrow in Fig. 9A) were closely associated with the GFRα1-IR terminal Schwann cells (arrowheads in Fig. 9A). In contrast to the other sets of mechanoreceptors studied, the longitudinal lanceolate terminal also had detectable, but weak, levels of GFRα1 protein (small arrowheads in Fig. 9B). Since neither longitudinal lanceolate ending nor Merkel cell-neurite complexes were affected in any GDNF mutant studied, GDNF is not vital for the survival of this class of primary sensory neurons or the formation of their specialized peripheral nerve endings. Nevertheless, the temporal and spatial expression of receptor and ligand at the target and at the terminal Schwann cells suggests that they might utilize GDNF during development.

DISCUSSION

We have shown a dynamic temporal and spatial regulation of gdnf, ntn and their putative ligand-binding receptor-units gfrα1, gfrα2 and gfrα3 within the target for specific classes of cutaneous primary sensory afferents. The expression correlates with the time and location of axonal invasion and/or sensory nerve ending formation. Moreover, we show for the first time that myelinated NGF-dependent cutaneous mechanoreceptors also require GDNF to develop. The arriving axon intimately follows the processes of terminal Schwann cells at the target which in turn highly express gfrα receptor subunits.

A role for GDNF and NTN in sensory nerve ending formation

The dynamic temporal expression of gdnf, ntn and their ligand-binding receptors within the FSC during embryonic and postnatal development correlated in time and location with the development of different sets of sensory nerve endings. Whereas ntn and gfrα2 expression correlated with the early development of Merkel cell-neurite complexes and Ruffini endings, gdnf and mainly gfrα1 expression correlated with the development of reticular endings, longitudinal and transverse lanceolate endings as well as the late formation of Merkel cell neurite complexes. The role of GDNF family members in chronological order of development of nerve endings will be discussed below.

At E13, several axons occupied the gfrα2-expressing mesenchyme surrounding the developing whisker follicle, which at this stage expressed both ntn and gfrα3. The low levels of ret in the tissue suggest that the target derived NTN might act on the arriving primary sensory axons.

Since the Merkel cell-neurite complexes are being developed over a fairly long period of time, spanning from about embryonic day 16 to the end of the first postnatal week, the expression of target derived neurotrophic factors at the early embryonic stage will differ from that at later postnatal stage.

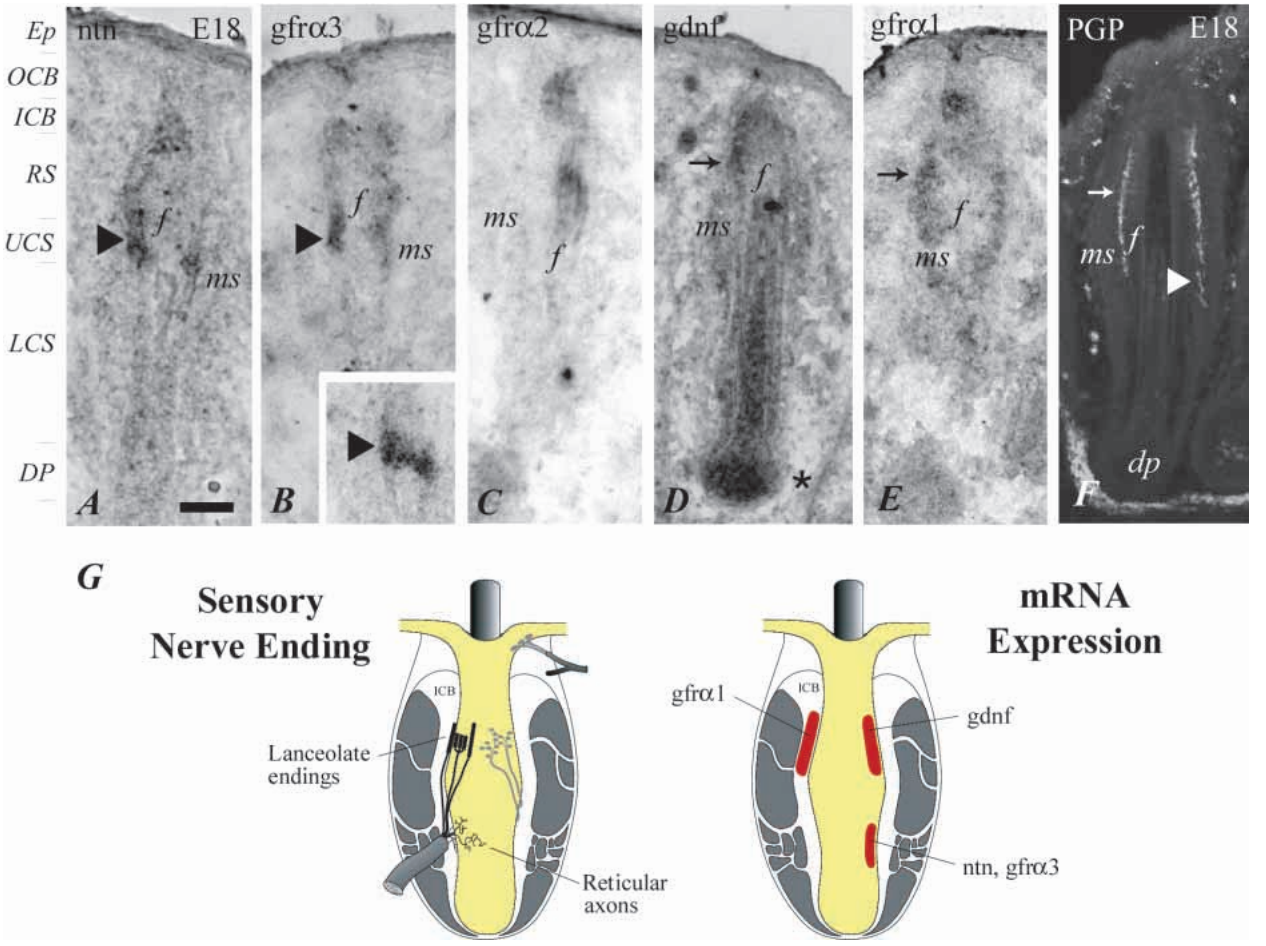

Fig. 6. Specific expression of gdnf and gfrα1 during the development of longitudinal lanceolate endings. (A-E) Expression of ntn (A), gfrα3 (B), gfrα2 (C), gdnf (D) and gfrα1 (E) in the whisker follicle (f) and the surrounding mesenchymal sheath (ms) at E18. The levels of the whisker follicles are indicated to the right: Epidermis (Ep); outer conical body (OCB); inner conical body (ICB); ring sinus (RS); upper cavernous sinus (UCS); lower cavernous sinus (LCS); dermal papilla (DP). The expression of ntn and gfrα3 (arrowheads), but not gfrα2 at the upper cavernous sinus is shown in A, B and C. Insert in B shows the strict localization of the gfrα3 mRNA to the upper cavernous sinus. This expression corresponds to the early invasion of the reticular axons (arrowheads in F,G). (D,E) Expression (arrows) of gdnf (D) in the outer root sheath at the level of ring sinus and gfrα1 mRNA (E) in the surrounding mesenchymal sheath (ms). This expression coincides with the development of the longitudinal lanceolate endings (see arrow in F). (F) Innervation of the whisker follicle at E18 visualized with anti-PGP 9.5. (G) Schematic summary diagram of the innervation and mRNA expression at E18. Scale bars, 100 μm.

At E16, ntn was transiently expressed in the whisker follicle strictly at the level of developing Merkel innervation, and gfrα2 mRNA was evident in the immediate surrounding mesenchyme. A few days later (E18), ntn and gfrα2 were down regulated whereas gdnf and gfrα1 were evident at the target. Moreover, in the postnatal mouse Merkel axons were closely associated with gfrα1-expressing terminal Schwann cells. Thus, our result suggests that later formed Merkel cell-neurite complexes do not have the same requirements for neurotrophic factors as those formed early. Such switch in dependency has previously been described for Merkel innervation in the FSC. For instance, Merkel neurons which depend on NT3 (Airaksinen et al., 1996; Fundin et al., 1997b) are also affected in NGF mutant mice (Fundin et al., 1997b) suggesting that Merkel innervation is sequentially depend on NT3 and NGF to develop. However, the absence of GDNF was not sufficient to affect the formation of Merkel cell-neurite complexes in our study. Thus, Merkel cell-neurite complexes might simultaneously depend on more than one neurotrophic factor, which has recently been shown for longitudinal lanceolate endings (Fundin et al., 1997b) and will be further discussed below, or that the utilization of GDNF is not associated with neuronal survival, axonal growth or ending formation. Nevertheless, the fact that ntn and gfrα2 were transiently expressed only at the early stage of Merkel cell-neurite complex formation suggests that NTN signaling might be involved in axonal guidance and/or the induction of Merkel ending formation. It is interesting to note that ntn expression was limited to the Merkel cell-neurite complexes at the outer root sheath and was not present at the rete ridge collar. A similar difference in dependency has been reported earlier for these two sets of Merkel innervation (Fundin et al., 1997b). While the absence of NGF affected the Merkel innervation at the level of ring sinus, the Merkel endings at the rete ridge collar developed normally.

There was detectable GFRα1-IR at the growth cones of the

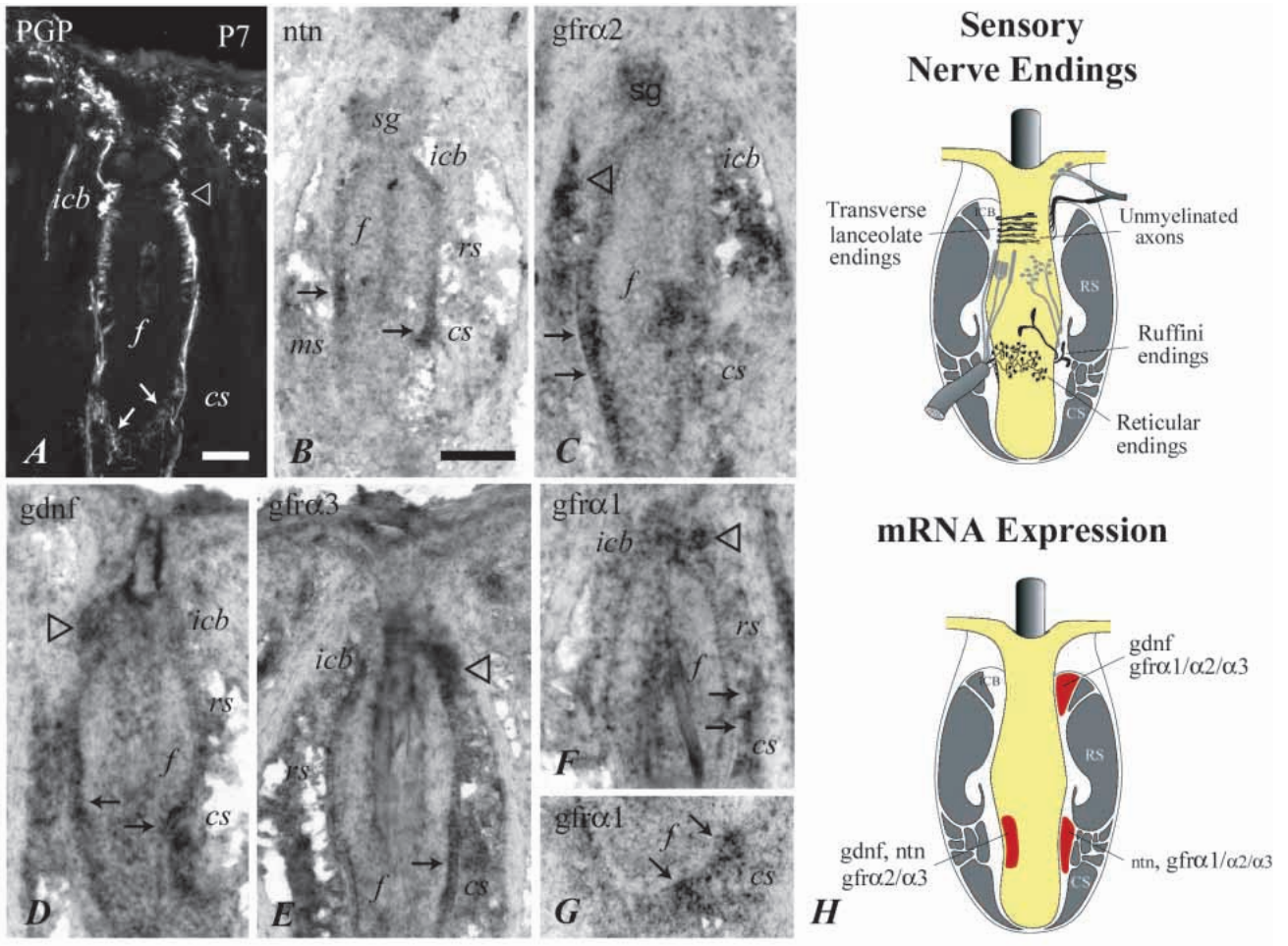

Fig. 7. The postnatal development of Ruffini, reticular and transverse lanceolate endings is matched by a region-specific expression of ntn and gdnf, respectively. The innervation and expression in the FSC at postnatal day 7 are illustrated in A-G and schematically shown in H (smaller letters indicates weaker mRNA expression). As revealed with anti-PGP 9.5 (A), a fairly developed set of reticular endings (arrows) at the level of cavernous sinus (cs) and the innervation at the inner conical body (icb; open arrowhead), which includes the myelinated transverse lanceolate ending are present at P7. The expression of GDNF-family ligands and receptors (shown in the upper left corner) are shown in B-G. Expression of ntn, gdnf, gfrα2 and gfrα3 (arrows in B-G) are all evident in the outer root sheath of the follicle at the level of cavernous sinus, which is the location for the developing reticular and Ruffini endings (A, H). In the surrounding mesenchymal sheath at this level, gfrα1 (arrows in F,G) and to a lesser degree gfrα2 and ntn are expressed. At the level of ICB, expression of gfrα1/α2/α3 as well as gdnf are evident (open arrowheads in C-F). Scale bar (100 μm), B applies for C-G.

axons that form longitudinal lanceolate endings at the same level of the FSC a few days later. However, these endings were not affected in any of the GDNF mutant mice. This class of mechanoreceptors is particularly interesting as they has been shown to depend simultaneously upon both NGF/trkA and BDNF/trkB signaling during development (Fundin et al., 1997b) and the deletion of either ligand/receptor does not alone result in a total loss of endings. Similarly, a reduction of GDNF might be compensated for by other trophic support in the GDNF mutant mice. Because all our other results indicate that GDNF is important for these neurons during their sensory nerve ending formation, we suggest, despite the fact that longitudinal lanceolate endings were not affected in GDNF mutant mice, that they might utilize GDNF during development.

In contrast to the longitudinal lanceolate ending discussed above, the reticular endings at the upper cavernous sinus level were detrimentally affected in adult GDNF$^{+/-}$ mice. This was further supported by postnatal expression of gdnf at the target of innervation. Moreover, we show that reticular endings do not depend on GDNF signaling prior to target innervation and that the utilization of GDNF is not associated with neuronal survival and axonal outgrowth but more specifically formation and maintenance of their peripheral nerve endings. Although reticular endings depend upon GDNF signaling, their axons lacked detectable GFRα1-IR. However, GFRα1 protein and mRNA were observed at the terminal Schwann cells that were closely associated with the arriving axons. Thus, either GDNF might signal through another receptor subunit, e.g. GFRα2 (Jing et al., 1997), to activate Ret present on the reticular axons, or GFRα1 produced by the terminal Schwann cells might be utilized. The latter hypothesis will be further discussed below.

The innervation to the ICB starts as late as postnatal day 3 and consists of several distinct classes of peptidergic/non-

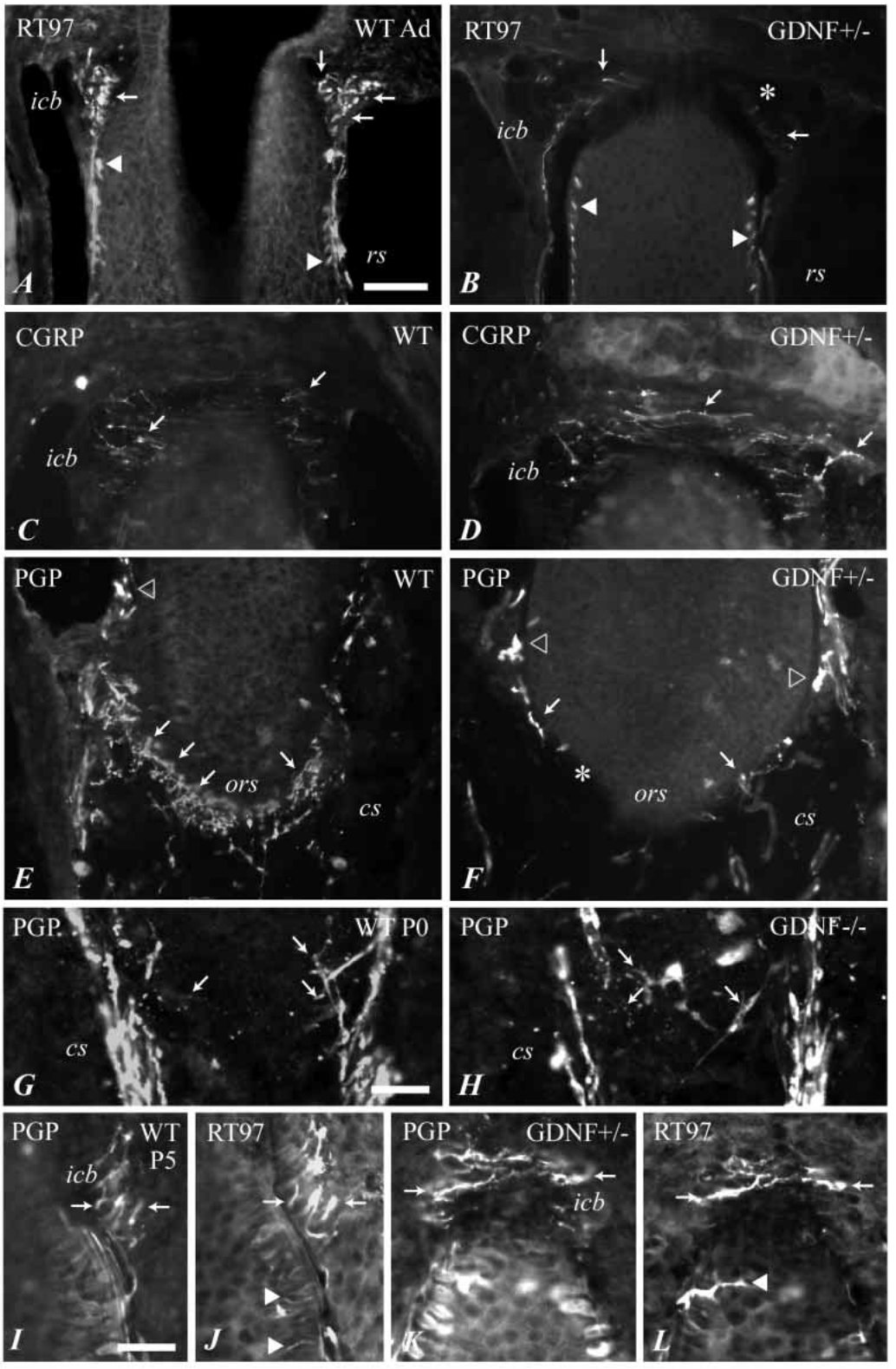

Fig. 8. The effect of GDNF gene deletion on the development of cutaneous innervation within the FSC studied with immunohistochemistry. A,C,E,G show adult wild-type mice, while B, D, F and H depict FSC innervation in the adult GDNF$^{+/-}$ mouse. (A,B) The reduction of transverse lanceolate endings (asterisk) is shown by a dramatic decrease of RT97-IR axons (arrows) in the inner conical body (icb). This loss was not evident among the sets of CGRP-IR axons (arrows in C and D) in the inner conical body. The Merkel terminals were not affected (arrowheads) (E,F) Anti-PGP 9.5 reveals the dramatic loss (asterisk in F) of reticular endings (small arrows) in the mesenchymal sheath at the level of cavernous sinus (cs). The Ruffini endings (outlined arrowheads) were not affected in the GDNF$^{+/-}$. Outer root sheath (ors). (G,H) Reticular axons were present in neonatal GDNF$^{-/-}$ mice (arrows in H) and were comparable with those observed in the wild-type mice of the same age (arrows in G). (I-L) Double-labeled sections showing the presence of transverse lanceolate axons in 5-day old (P5) GDNF$^{+/-}$ mice (arrows in K and L). As revealed with RT97, the transverse lanceolate axons appeared normal in P5 GDNF$^{+/-}$ mice (arrows in L, compare J). Merkel terminals (arrowheads) were not affected at this age. Scale bar (100 μm) A-F; (25 μm) G and H; (50 μm) I-L.

peptidergic, *Griffonia simplicifolia* isolectin I-B4 (IB4) positive/IB4 negative unmyelinated axons as well as a myelinated RT97-IR mechanoreceptor, i.e. transverse lanceolate ending (Fundin et al., 1994; Rice et al., 1997, 1993). All sets of ICB axon depend upon NGF/trkA signaling prior to their target invasion (Fundin et al., 1997b). In contrast, only the myelinated RT97-IR transverse lanceolate endings were detrimentally affected in the adult GDNF$^{+/-}$ mice. Similar to reticular endings, transverse lanceolate endings depend upon GDNF signaling following target innervation. Thus, GDNF seem to be involved in the postnatal formation and maintenance of different classes of NGF-dependent

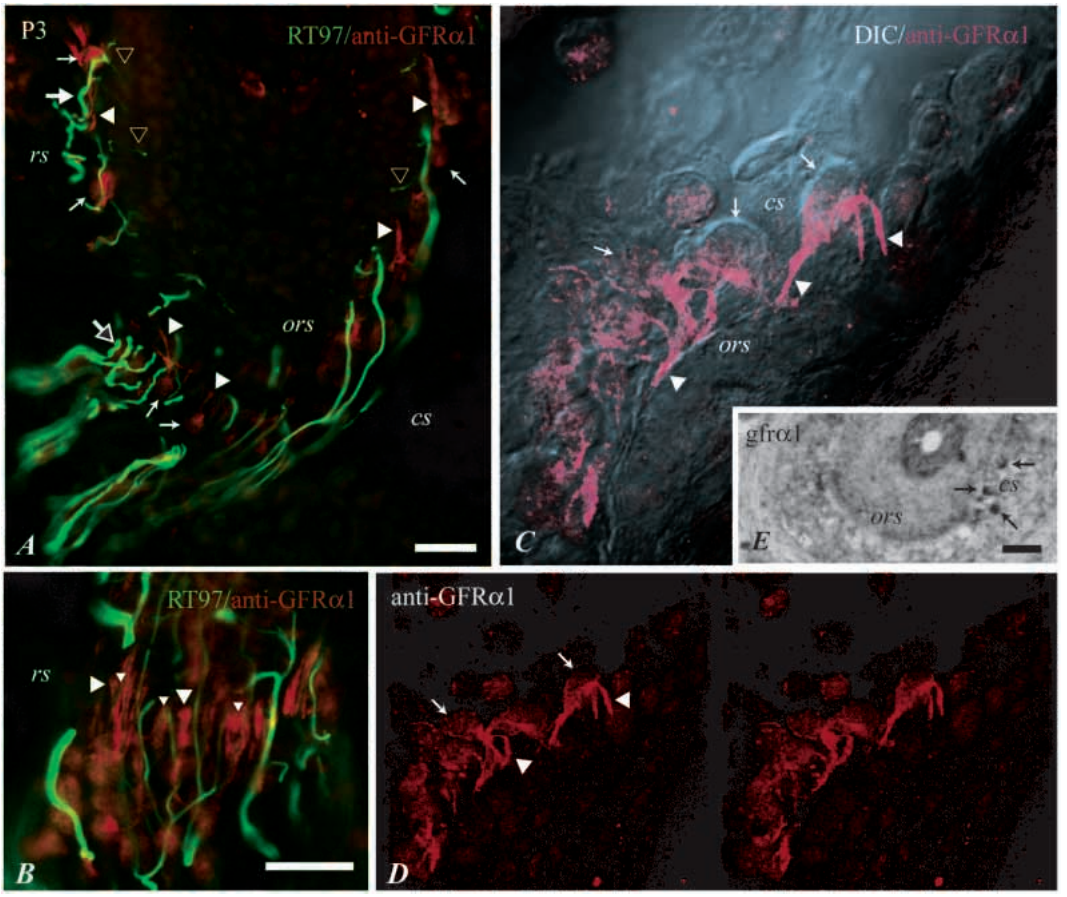

Fig. 9. GFRα1 protein and mRNA is primarily located in the terminal Schwann cells at the target. (A,B) Double-labeled images showing RT97-IR (green) and anti-GFRα1-IR (red) in the FSC at P3. The terminal Schwann cells (small arrows in A, C and D) and their processes (large arrowheads in A-D; note the apical density) are GFRα1-IR, whereas the RT97-IR axons that form reticular endings (large outlined arrow in A) as well as Merkel cell-neurite complexes (large arrow in A indicate Merkel axons; outlined arrowheads in A indicate Merkel terminals) do not show detectable GFRα1-labeling. In contrast, the short and highly branched form of longitudinal lanceolate terminals are GFRα1-IR (small arrowheads in B). (C,D) Confocal images depicting the localization of GFRα1 protein in the terminal Schwann cells (arrows) and their processes (arrowheads). (C) A stack of 61 optical sections are projected on a DIC-image to visualize the distribution of GFRα1 at the terminal Schwann cells. (D) A stereoprojection made from the same stack of optical sections obtained in C. (E) Terminal Schwann cells located in the mesenchymal sheath (ms) expressing gfrα1 mRNA (arrows). Outer root sheath (ors). Scale bar (A,B and E) 50 μm, (C) 10 μm and (D) 20 μm.

mechanoreceptors. This dependency was further confirmed by expression of gdnf at the target during the first postnatal weeks. Interestingly, all receptor subunits were expressed at the target at the time of axonal ingrowth and ending formation. The significant function of this multi-receptor expression in the ICB remains to be elucidated.

In contrast to the total lack of ICB innervation observed in NGF mutant mice, the CGRP-IR innervation to the ICB was not affected in GDNF mutant mice, which is in agreement with previous results showing that the axotomized adult CGRP-IR primary sensory neurons were rescued by NGF, but not GDNF (Bennett et al., 1998; Leclere et al., 1998). Interestingly, in $GDNF^{+/-}$ mice we found no loss of unmyelinated innervation to the ICB, which includes a large proportion of IB4-positive axons (Rice et al., 1997). This result was somewhat surprising since this class of neurons has been shown to express GFRα1 and retrogradely transport GDNF postnatally (Molliver et al., 1997). Moreover, GDNF significantly increased the numbers of outgrowing axons labeled with IB4 in adult DRG explant culture and could further prevent several axotomy-induced changes in these neurons (Bennett et al., 1998; Leclere et al., 1998). Thus, our results suggest that GDNF is not crucial for the normal development of IB4-positive neurons, but might instead play an important role in the regeneration process in the adult animal.

Are GDNF and NTN involved in skin morphogenesis?

GDNF is crucial during kidney morphogenesis (for review see, Sariola and Sainio, 1997). During this period, Ret is expressed in the nephrogenic bud (Nosrat et al., 1997) whereas GDNF is expressed in the condensing mesenchyme (Hellmich et al., 1996; Nosrat et al., 1996; Suvanto et al., 1996; Trupp et al., 1995). Consistent with an inductive role during kidney development, GDNF mutant mice fail to develop kidneys (Moore et al., 1996; Pichel et al., 1996; Sanchez et al., 1996). Specific patterns of gdnf and ntn mRNA during whisker follicle induction opened up the possibility of a role also in skin morphogenesis. First, during the early invagination of epidermis to form the whisker follicles at E11, ntn and gfrα3 were expressed throughout the mesenchyme, while punctuate patterns of gdnf could be detected in the epidermis. Second, at E13 ntn was densely expressed in the whisker follicles, whereas gfrα2 was detected in the immediately surrounding mesenchyme. Third, all ligands and receptors studied were highly expressed from E16 to P14 in the growth zone of the whisker follicle, i.e. the outer root sheath adjacent to the inductive dermal papilla (see Fig. 1). However, the very low levels of ret expressed in the whisker pad at E11 and E13 argues against a role of GDNF and NTN in whisker follicle

induction. Since the reduction of GDNF in the GDNF mutant mice did not result in any obvious distortion of skin development, GDNF alone may not be crucial for skin development. However, this issue will have to be further analyzed in GDNF/NTN compound null mutant mice.

Regulation of GFRα3 expression suggests a role in NTN receptor interactions

Based upon the pattern of expression in the target tissue, GFRα2 seem to be mainly involved in NTN signaling. However, our results also indicate that GFRα3 may be associated with NTN action. It should be noted that the homology between GFRα3 and GFRα1, and GFRα3 and GFRα2 is significantly lower (32% and 37%, respectively) than between GFRα1 and GFRα2 (50%). Thus, GFRα3 being a more distant relative suggests that it could play different roles than GFRα1 and GFRα2. Consistent with this notion are recent findings showing that whereas GFRα3 is unable to activate Ret in conjunction with NTN or GDNF in fibroblasts (Baloh et al., 1998a; Worby et al., 1998), it can mediate a crosslinking of GDNF to Ret when both receptors were coexpressed in COS cells (Trupp et al., 1998).

Our finding that gfrα3 co-localize with ntn at the target, i.e. the outer root sheath, just prior to the ingrowth of Merkel axons suggests a possible interaction of GFRα3 with NTN signaling. Because it does not activate Ret but still can interact with the receptor it is conceivable that it could modulate Ret activation by suppressing the action of NTN. Furthermore, during Merkel cell-neurite formation ntn and gfrα3 were expressed in the outer root sheath just below the Merkel target. This portion of the FSC normally contains a small number of Merkel cells during development which gradually disappear and rarely gets innervated by Merkel axons. Thus, it seems possible that GFRα3 might antagonize the action of NTN by associating with NTN and Ret without activating a signaling cascade and thereby regulating the timing and perhaps distribution of the developing Merkel innervation. It is also possible that the coexpression of NTN and GFRα3 is coincidental and the newly discovered ligand for GFRα3, artemin (Baloh et al., 1998b), might be involved in stimulating and/or suppressing the development of specific sets of sensory nerve endings by signaling through GFRα3.

NGF-dependent classes of myelinated cutaneous mechanoreceptors switch to GDNF dependency

In order to understand the functional significance of GDNF in the development of cutaneous innervation, we studied homozygous and heterozygous mice carrying a deletion in the GDNF gene. In contrast to homozygous GDNF null mutant mice, which die at birth, the heterozygotes are viable into adulthood and all sets of cutaneous innervation could therefore be studied in detail.

In newborn GDNF null mutated mice the whisker innervation was comparable to that of the wild-type mice, which is similar to what have earlier been suggested by Granholm et al. (1997). In addition, we show a detrimental effect upon this innervation in adult heterozygous GDNF mutant mice. Although the half quantity of GDNF could still be secreted in those mice, two sets of RT97-IR myelinated mechanoreceptors, i.e. reticular endings and transverse lanceolate endings, were severely affected. As shown earlier, the development of these sets of cutaneous sensory nerve endings are totally dependent upon NGF/trkA signaling prior to target invasion (Fundin et al., 1997b). Here we show that these sets of mechanoreceptors also require GDNF signaling, presumably through GFRα1. Since absence of GDNF failed to affect neuronal survival and axonal outgrowth and that gdnf was expressed at the target during their ending formation, GDNF seems to play an important target-derived role in the process of nerve end formation as well as the maintenance of these sets of mechanoreceptors. Consistent with our in vivo findings, Leclere et al. (1998) has recently reported that culturing adult DRG neurons with GDNF results not only in outgrowth of IB4-positive unmyelinated profiles as described previously for early postnatal neurons (Molliver et al., 1997), but also RT97-IR axons.

Terminal Schwann cells as a local source of GFRα1

GPI anchors are sorting signals which encode information on the destination of the protein within the cell (for review see, Faivre-Sarrailh and Rougon, 1997). In polarized epithelial cells, GPI-anchored proteins are selectively sorted from golgi via coclustering with glycolipids (Brown and Rose, 1992) to the apical surface of the cells (Lisanti and Rodriguez-Boulan, 1990). This sorting mechanism is also present in other cells such as neurons (Dotti et al., 1991), in which the GPI-anchored proteins are targeted to the axons where they may be further segregated into membrane microdomains (Anderson, 1993). It is interesting that the GDNF family of receptors is the only GPI anchored family of receptors associated with a tyrosine kinase receptor. The significance of GPI-anchored receptors for the function of GDNF and NTN is however not yet known.

During axonal growth, proteins localized to the growth cone are involved in the choices between different directions of elongation as well as in axonal termination by interactions with environmental signals. Terminal Schwann cells have long finger-like processes apically. Because of the intimate relation with the axon, these processes are likely to guide the arriving axon to their correct location and finally participate in the formation of nerve endings, as has been shown at the neuromuscular junction (Son and Thompson, 1995a; Son and Thompson, 1995b). Thus, the intimate relation between GDNF-family ligand binding receptors and Ret present on terminal Schwann cells and axons, respectively, indicates an essential role for GDNF and NTN during nerve ending formation.

Our results show that gfrα1 expressed by terminal Schwann cells is located to their apical surface and their long processes. Since most primary sensory neurons express ret during development, it is tempting to speculate that GFRα1 might be released from the surface of the terminal Schwann cells to form a GDNF-GFRα1 complex that finally bind and activate Ret at the surface of the distal axon/ending, and thereby allow a very strict local effect of GDNF. This hypothesis is supported by the fact that GDNF and NTN in vitro can activate Ret in the presence of soluble GFRα1 and GFRα2 (Jing et al., 1996; Klein et al., 1997; Treanor et al., 1996). Interestingly, several GPI molecules have been postulated to have dual effects depending on whether they are in a soluble form or anchored to a membrane (for review see, Faivre-Sarrailh and Rougon, 1997). For instance, the glycoprotein F3, which is found predominantly at the axons in the mouse, is able to induce

inhibition of neurite growth when immobilized at the membrane (Buttiglione et al., 1996), whereas soluble F3 promotes neurite outgrowth (Durbeck et al., 1994; Durbeck et al., 1992). Thus, it is possible that the GFRαs may elicit various effects on different cell populations depending on whether they are soluble or GPI anchored to the membrane. On the other hand, Yu et al. (1998) showed that an immobilized GDNF/GFRα1/Fc complex on cells could activate Ret expressed on the surface of another Neuro-2a cell. This phenomenon, termed 'trans activation', might in turn explain the lack of GFRα1-IR in the axons and termini of the reticular ending revealed in this study. Thus, GFRα1 might not be released from the terminal Schwann cells, but instead, as GPI-anchored receptors present on these cells, activate Ret located only at the closely associated axons. The reticular endings present in adult $GDNF^{+/-}$ mice display only a few branches compared to the elaborate endings present in wild-type mice. Thus, the intricate interaction between the processes of terminal Schwann cell and the growth cones of the ingrowing axons might provide an important substrate for the exact axonal termination and specific formation of the sensory nerve endings.

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