

Positive and negative interactions of GDNF, NTN and ART in developing sensory neuron subpopulations, and their collaboration with neurotrophins

Christel Baudet¹, Åsa Mikaelis¹, Heiner Westphal², Jens Johansen³, Teit E. Johansen³ and Patrik Ernfors^{1,*}

¹Laboratory of Molecular Neurobiology, Medical Biochemistry and Biophysics, Karolinska Institutet, S-171 77 Stockholm, Sweden

²Laboratory of Mammalian Genes and Development, National Institute of Health, Bethesda, MD 20892, USA

³NsGene A/S, DK-2570 Ballerup, Denmark

*Author for correspondence (e-mail: patrik@cajal.mbb.ki.se)

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SUMMARY

Glial cell line-derived neurotrophic factor (GDNF), neurturin (NTN) and neublastin/artemin (ART) are distant members of the transforming growth factor β family, and have been shown to elicit neurotrophic effects upon several classes of peripheral and central neurons. Limited information from *in vitro* and expression studies has also substantiated a role for GDNF family ligands in mammalian somatosensory neuron development. Here, we show that although dorsal root ganglion (DRG) sensory neurons express GDNF family receptors embryonically, they do not survive in response to their ligands. The regulation of survival emerges postnatally for all GDNF family ligands. GDNF and NTN support distinct subpopulations that can be separated with respect to their expression of GDNF family receptors, whereas ART supports neurons in populations that are also responsive to GDNF or NTN. Sensory neurons that coexpress GDNF

family receptors are medium sized, whereas small-caliber nociceptive cells preferentially express a single receptor. In contrast to brain-derived neurotrophic factor (BDNF)-dependent neurons, embryonic nerve growth factor (NGF)-dependent nociceptive neurons switch dependency to GDNF, NTN and ART postnatally. Neurons that survive in the presence of neurotrophin 3 (NT3) or neurotrophin 4 (NT4), including proprioceptive afferents, Merkel end organs and D-hair afferents, are also supported by GDNF family ligands neonatally, although at postnatal stages they lose their dependency on GDNF and NTN. At late postnatal stages, ART prevents survival elicited by GDNF and NTN. These data provide new insights on the roles of GDNF family ligands in sensory neuron development.

Key words: GDNF, NTN, ART, Neurotrophin, DRG, Mouse

INTRODUCTION

The GDNF family of ligands consists of four members: GDNF (Lin et al., 1993), NTN (Kotzbauer et al., 1996), persephin (PSP; Milbrandt et al., 1998) and ART (Baloh et al., 1998a). They all activate a common signaling component, the receptor tyrosine kinase Ret (Durbeck et al., 1996; Jing et al., 1996; Treanor et al., 1996; Trupp et al., 1996). The ligand specificity is achieved by the preferential binding to a co-receptor, which belongs to a novel class of glycosylphosphatidyl inositol membrane-linked proteins called GDNF family receptor alpha (GFR α). Four distinct GFR α s have been described, GFR α 1 to 4, that respectively bind preferentially to GDNF, NTN, ART and PSP (Klein et al., 1997; Buj-Bello et al., 1997; Creedon et al., 1997; Baloh et al., 1997, 1998a,b; Jing et al., 1997; Sanicola et al., 1997; Naveilhan et al., 1998; Cacalano et al., 1998; Enomoto et al., 1998; Rossi et al., 1999; Enokido et al., 1998; Worby et al., 1998; Heuckeroth et al., 1999; Nishino et al., 1999). GFR α 1-3 have been identified in mammals, but so far GFR α 4 has only been identified in chick (Enokido et al., 1998). The preferred ligand/co-receptor combinations have mostly been studied *in vitro* but the recent comparison between

mice deficient for GDNF or NTN with the mice deficient for one of the GFR α s prove these pairings to be biologically relevant.

The GDNF family ligands share many biological properties *in vitro*, including the potent survival-promoting effects on midbrain dopaminergic neurons (Lin et al., 1993; Horger et al., 1998; Baloh et al., 1998a; Milbrandt et al., 1998). However, GDNF, NTN and ART, but not PSP, are able to support the survival of peripheral neuronal populations *in vitro* (Buj-Bello et al., 1995; Baloh et al., 1998a; Milbrandt et al., 1998; Heuckeroth et al., 1999; Forgie et al., 1999). Although their roles on enteric, sympathetic and parasympathetic neurons have been analyzed in detail in culture studies and null mutant mice, much less is known of their effects on sensory neurons (Moore et al., 1996; Sanchez et al., 1996; Hearn et al., 1998; Heuckeroth et al., 1998, 1999). In GDNF-deficient mice there is a 23% reduction of the number of neurons in the dorsal root ganglion (DRG) at P0, and in newborn mice lacking NTN, 45% of the GFR α 2-expressing cells are lost (Moore et al., 1996; Heuckeroth et al., 1999). *In vitro* data show that GDNF and NTN have a survival-promoting effect of less than 10% in embryonic day (E) 15 rat DRG sensory neurons (Kotzbauer et

al., 1996; Matheson et al., 1997). In contrast, GDNF has been found to support the survival of a group of newborn rat neurons belonging to the isolectine B4 (IB4)-positive subpopulation, which constitutes 35% of the total DRG neuronal population (Matheson et al., 1997; Bennett et al., 1998). Like GDNF and NTN, ART supports the survival of only a proportion of P1 rat DRG neurons in culture and in situ hybridization has revealed a high expression of GFR α 3 in perinatal DRG (Baloh et al., 1998a; Widenfalk et al., 1998).

We have previously analyzed GFR α 1, GFR α 2 and GFR α 3 expression in the trigeminal ganglion (Naveilhan et al., 1998). However, little is known of the relationship between cytochemical properties, soma size and physiology of the trigeminal ganglion neurons. In contrast to trigeminal ganglion neurons, the subpopulations of DRG neurons subserving different sensory modality responsiveness, and displaying different cytochemical properties and soma sizes have been identified and extensively characterized (Carr and Nagy, 1993). Large-diameter DRG cells mostly possess myelinated axons and respond principally to low-threshold stimuli, whereas small-diameter DRG cells have unmyelinated axons and respond to nociceptive and thermoceptive stimuli, finally, intermediate-size neurons contain mechanoreceptive afferents. Extensive studies of the neurotrophins have elegantly established that specific subsets of DRG neurons mediating different sensory modalities are supported by particular members of the neurotrophin family in a dynamic manner during development (reviewed by Lewin and Barde, 1996; Snider and Wright, 1996). The majority of rodent DRG neurons, including all small pain transducing neurons, express TrkA and require nerve growth factor (NGF) for survival during embryonic development; but half of the small neurons (those that can bind the lectin IB4) downregulate TrkA during the three weeks after birth. Molliver et al. (1997) have shown that these particular neurons undergo a shift in receptor tyrosine kinase expression from TrkA to Ret, suggesting a switch from NGF to GDNF responsiveness during development.

Limited information is available on GDNF family ligands during embryonic and postnatal sensory neurons development, especially for ART. In this study we have characterized the survival effect of three GDNF family ligands (GDNF, NTN, ART) through embryonic and postnatal development, and have analyzed the expression of their receptors in wild-type and GDNF-null mutant mice.

MATERIALS AND METHODS

Animals

All experiments were performed on C57/Bl6 mice. Embryos were studied at E12, E13 or E16. Postnatal animals were studied at postnatal days 0 (P0), P7 or P15. The day of the vaginal plug was considered as E0.

Neuronal survival assay

For each experiment, approximately 100 DRG from E12, E16, P0, P7 or P15 animals were dissected out and collected in PBS/glucose. In order to dissociate the neurons, the DRGs were incubated at 37°C with trypsin/DNase 0.05% (Gibco/BRL/Sigma). When dissociating ganglia from P0 to P15 pups, a prior treatment with collagenase/dispace (1 mg/ml; Boehringer Mannheim) was carried

out. After removal of the trypsin solution, the ganglia were washed once with DMEM/10% heat-inactivated horse serum and twice with defined medium (see below). The ganglia were then gently triturated with a fire-polished Pasteur pipette to give a single-cell suspension. Non-neuronal cells were eliminated by preplating. The neurons were plated on 24-well plates (Nunc) precoated with polyornithine (0.5 mg/ml, 30 minutes; Sigma) and laminine (20 μ g/ml, overnight; Gibco/BRL) in a defined medium consisting of Ham's F14 supplemented with 2mM glutamine, 0.35% bovine serum albumin, 60 ng/ml progesterone, 16 μ g/ml putrescine, 400 ng/ml L-tyroxine, 38 ng/ml sodium selenite, 340 ng/ml triiodo-thyronine, 60 μ g/ml penicillin and 100 μ g/ml streptomycin. The primary cultures were maintained for 48 hours at 37°C in a humidified incubator under 5% CO₂. After 4 hours of incubation, the neurons were clearly recognized by their bipolar morphology under phase-contrast optics and the number of plated neurons was counted. The data illustrated are compiled from three experiments, each performed in triplicate.

GDNF (Promega) and NTN (Peprotech) were used at 10 ng/ml. To investigate the survival effect of ART, we used conditioned medium from HiB5 cells transfected with ART (Rosenblad et al., 2000). Serum-free DMEM medium was conditioned for 48 hours. The conditioned medium was used at a dilution of 1:800, which was determined by testing different dilution of the conditioned medium on E12 and P7 primary cultures of DRG neurons (data not shown) and choosing the highest dilution resulting in maximal survival effects. NGF (Promega), BDNF, NT3 and NT4 (gift from Regeneron Pharmaceutical) were used at 10 ng/ml. The control conditions were obtained by cultivating the neurons in absence of any growth factor or in presence of conditioned medium from HiB5 non-transfected cells when working with ART. No survival effects were detected in conditioned medium from HiB5 non-transfected cells and no adverse effects were seen of this control medium on cultures with GDNF or NTN.

Probes

The rat GFR α 1 and mouse Ret probes used in the DIG in situ hybridization have been described earlier (Naveilhan et al., 1997) and correspond to nucleotides 862-1160 (Jing et al., 1996) and 1381-1750 (Iwamoto et al., 1993), respectively. The mouse GFR α 2 and GFR α 3 cDNA probes correspond to nucleotides 805-1215 (Baloh et al., 1997) and 601-910 (Naveilhan et al., 1998). These cDNAs were used to prepare transcripts labeled with digoxigenin-UTP (DIG-UTP), as described in Naveilhan et al., 1998.

In situ hybridization procedure

For in situ hybridization, tissues of time-staged mouse E13, E16 and postnatal day (P) 0 were fresh frozen, positioned on a metal block and sectioned transversely (14 μ m) on a Leitz cryostat. All sections were thaw-mounted onto slides pretreated with 3-aminopropyl triethoxysilane (Sigma) and kept frozen until hybridization. Before use, the sections were dried at room temperature for 30 minutes, fixed in 4% paraformaldehyde for 15 minutes, washed with 1 \times PBS and distilled water. Then a deproteinization step was performed in 0.1 M HCl for 15 minutes and, after two washes with 1 \times PBS, the slides were incubated 20 minutes with 0.25% acetic anhydride in 0.1 M triethanolamine. The slides were prehybridized for 4 hours in hybridization buffer (50% formamide, 5 \times sodium saline citrate (SSC), 1mg/ml yeast tRNA, 1 \times Denhardt's solution, 0.1% Tween-20, 0.1% CHAPS, 5 mM EDTA) at 60°C before an overnight incubation with the probe (40 ng/slide in 200 μ l hybridization buffer) at the same temperature. The slides were then washed as follows: a quick wash in 5 \times SSC (60°C), two 15 minutes washes in 2 \times SSC (60°C and 37°C, respectively), a 30 minutes treatment with 0.2 μ g/ml RNase A in 2 \times SSC at 37°C followed by a 15 minutes wash with 1 \times PBS/0.1% Triton X-100 (60°C) and a last wash with PBT (1 \times PBS, 0.1% Triton X-100, 2mg/ml bovine serum albumin) for 15 minutes at room temperature. The slides were incubated in PBT/10% heat inactivated

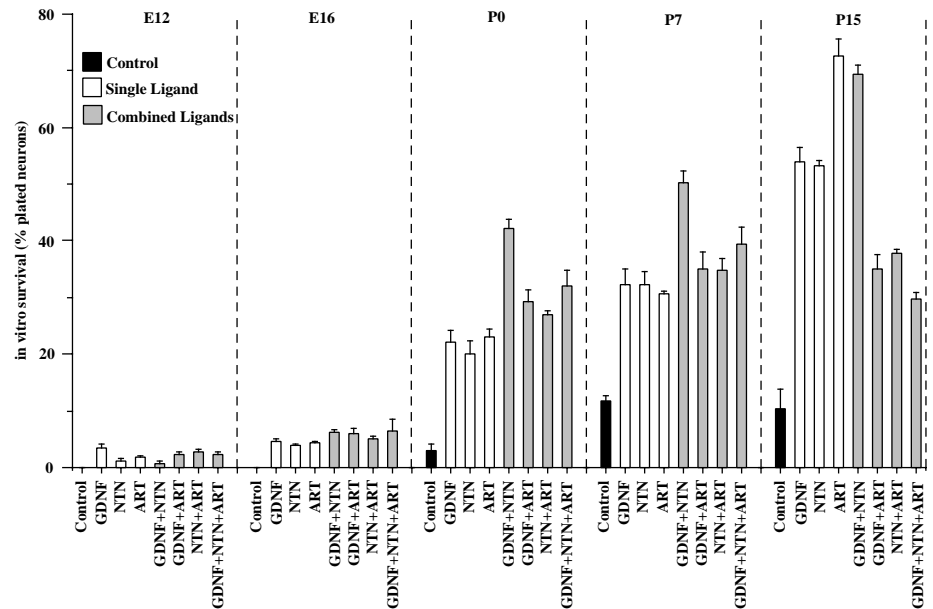


Fig. 1. GDNF, NTN and ART dependency during DRG development. The graph represents the percentage of surviving DRG neurons 48 hours after plating in the presence of the indicated factors. GDNF and NTN were added at 10ng/ml to the culture. ART conditioned medium was diluted at 1/800. For each developmental stage (E12, E16, P0, P7, P15), the experiments were conducted in triplicate and repeated at least three times (mean and s.e.m., $n=9-15$).

goat serum (PHIGS) for 5 hours at room temperature prior to the overnight incubation at 4°C with anti-DIG antibody (Boehringer Mannheim, 1:2000 in PHIGS). The post antibody washes were performed in 1×PBS/0.1% Triton X-100, twice for 15 minutes, then once for 30 minutes at room temperature followed by a 5 minutes incubation in alkaline phosphatase buffer (100 mM Tris pH 9.5, 50 mM MgCl₂, 100 mM NaCl, 0.1% Tween 20) containing 5mM levamisol (Sigma) (AP-L). The visualization occurred by mixing 3.5 μl NBT (nitro blue tetrazolium, Boehringer Mannheim) and 3 μl BCIP (5-bromo-4-chloro-3-indoyl phosphate, Boehringer Mannheim) per ml of AP-L. After 24 to 72 hours of incubation, when the staining was satisfactory, the slides were washed in 1×PBS and mounted in PBS:glycerol (1:9). The total number of DRG neurons and the receptor-expressing neurons were determined as previously described (Elshamy and Enrnfors, 1996).

Size-frequency analysis

Embryos were serially sectioned and collected on alternating slides in eight series. Each series were hybridized by nonradioactive in situ hybridization with each probe, (i.e. Ret, GFR α 1, GFR α 2 and GFR α 3) and one section was stained with Cresyl Violet. Approximately 200 labeled DRG neurons with a visible nucleus were captured using a 20× objective (control mice $n=8$ DRG, GDNF-null mutant mice $n=8$ DRG) and the images were processed and analyzed for soma area determination using the IP-Lab spectrum software.

Genotyping of GDNF mutant mice

Heterozygous GDNF mutant mice were bred, and their offspring were collected at birth and used for the experiments. All of the neonatal mice were genotyped for wild-type and GDNF mutant alleles by PCR as previously described (Pichel et al, 1996).

RESULTS

Survival of mouse embryonic and postnatal DRG neurons by GDNF family ligands

Previous results have shown that GDNF poorly supports the survival of E15 rat DRG neurons but does promote the survival of 35% of the neonatal DRG neurons (Matheson et al., 1997). NTN and ART have similarly been shown to support the survival of neonatal DRG neurons but their roles in embryonic

development and at later postnatal stages are unknown (Kotzbauer et al., 1996; Baloh et al., 1998a). Since PSP seems not to support the survival of sensory neurons, in this study we have focused on the developmental role of the other three GDNF family members from early embryonic stages to two weeks after birth in glia-free cultures of DRG neurons using a defined medium. The neurons were plated at low density in the presence of GDNF, NTN or ART and counted after 48 hours of incubation (Fig. 1). Our results show that less than 5% of the embryonic neurons respond to GDNF, NTN and ART by survival. In contrast, a robust survival response to GDNF, NTN and ART was seen at birth and the number of surviving neurons increased progressively to P15. We observed a survival of approximately 20% of the plated neurons at birth for all three ligands, whereas at P15 it had reached 55% in presence of GDNF or NTN, and 72% in presence of ART.

We then examined whether GDNF, NTN and ART supported the same or different subsets of DRG neurons. For that purpose, we cultured E12, E16, P0, P7 and P15 DRG neurons in presence of all combinations of ligands (Fig. 1). No significant increased survival was observed with any combination of two or all three GDNF family ligands on embryonic neurons. However, after birth, the survival effect of GDNF was additive to that of NTN, indicating that the two ligands support two different subpopulations of neonatal DRG neurons. At later stages, the survival effect of GDNF and NTN combined was not fully additive compared with the effect of the ligands alone, suggesting that some of the cells responding to GDNF may also respond to NTN at these stages. However, this combination of GDNF family members was the only one leading to additive survival. Combining GDNF/ART, NTN/ART and GDNF/NTN/ART on P0 and P7 neurons did not lead to any increased survival compared with the treatment with a single ligand. Interestingly, at P15, the combination of GDNF/ART, NTN/ART and GDNF/NTN/ART all led to significantly reduced survival when compared with cultures grown with GDNF, NTN or ART only. Thus, ART in any combination with other GDNF family ligands lead to significantly reduced survival.

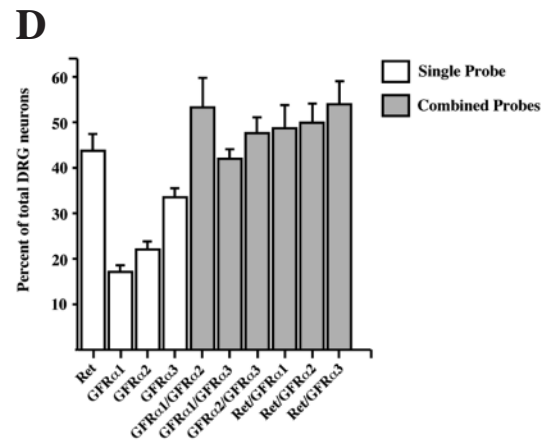
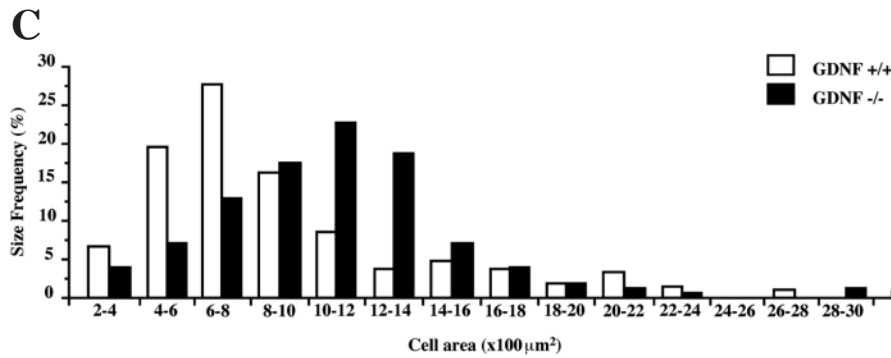
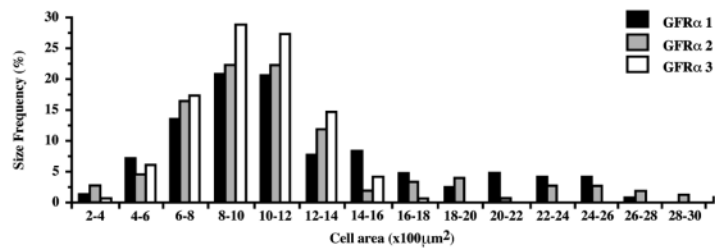
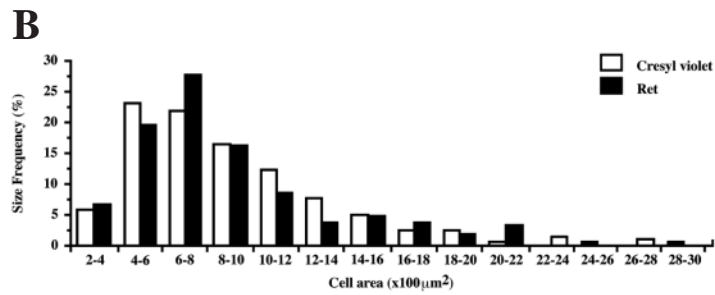
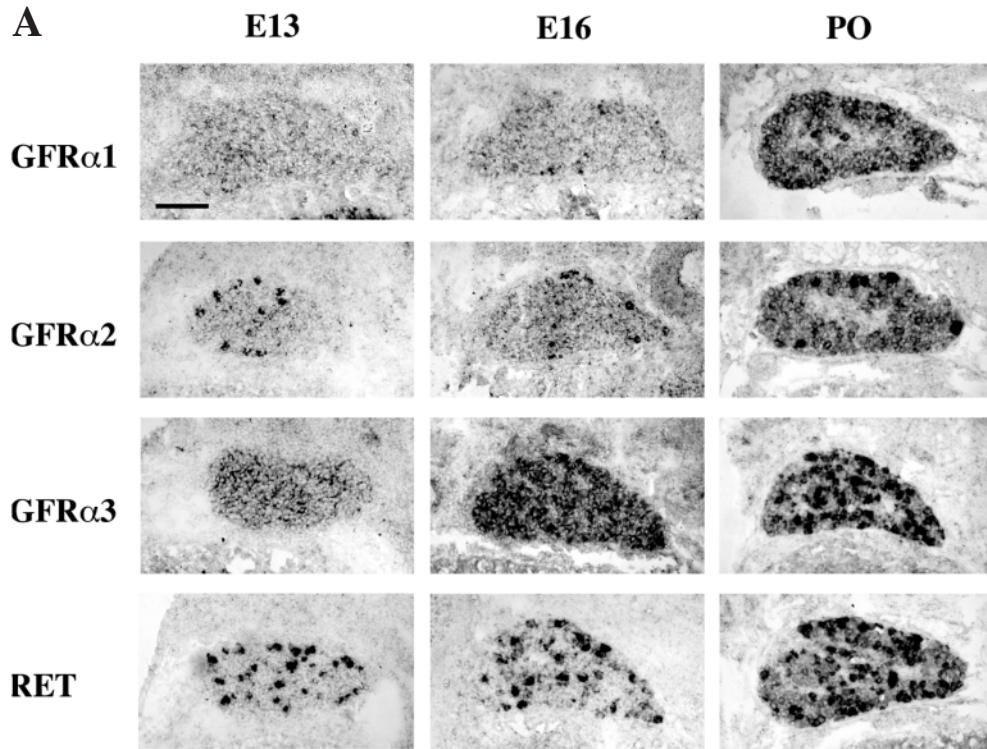


Fig. 2. (A) Expression of GFR α receptors and Ret mRNAs during embryonic and postnatal development. A nonradioactive in situ hybridization was performed on sectioned murine lumbar DRGs at E13, E16 and P0 for the detection of GFR α 1, GFR α 2, GFR α 3 and Ret transcripts. (B) Cell size-related expression of the different GDNF family receptors in lumbar DRG neurons. The size frequency histograms show the percentage of neonatal DRG neurons labeled by the probes for GFR α 1, GFR α 2, GFR α 3 and Ret within each indicated soma area. Each cell size spectrum can be compared with the general size distribution in a Cresyl Violet-stained ganglion. (C) Size frequency analysis of neonatal lumbar DRG neurons expressing Ret in newborn GDNF null mutant mice compared with wild-type animals. (D) Combined labeling of GFR α 1-3 and Ret receptors (gray bars) compared with the single probe labeling (white bars) in DRG neurons. Sections of neonatal mouse lumbar DRG were hybridized with either a single probe or a cocktail of two probes as indicated on the graph. The number of labeled neurons was quantified and the total number of neurons in the DRG was counted on adjacent Cresyl Violet-stained sections. The percentage of neurons labeled for each receptor(s) was calculated (mean \pm s.e.m.). Scale bar: 170 μ m at P0; 100 μ m at E16 and 70 μ m at E12.

Expression and developmental regulation of the four GDNF family receptor components (GFR α 1-3 and Ret)

Nonradioactive in situ hybridization was used to visualize the expression of the different GDNF family receptor components at different developmental stages in mouse lumbar DRG (Fig. 2A). Ret mRNA was clearly expressed in many neurons at E13 and E16, but the number of Ret mRNA-expressing cells had increased markedly at birth (24.4 \pm 1% and 44 \pm 3.5% of the total number of cells at E16 and P0, respectively). The temporal sequence of GFR α 1 and GFR α 2 expression began with low expression at the early stage (E13) towards a much higher expression at birth. In contrast, the expression of GFR α 3 was low in most neurons at E13 but the levels had increased at E16 in most neurons of the ganglion. GFR α 3 mRNA remained high at birth, but was localized to only a subpopulation of neurons.

In a size frequency measurement of the DRG neurons expressing Ret, GFR α 1, GFR α 2 or GFR α 3 mRNAs at birth (Fig. 2B), Ret was expressed in small, intermediate and large neurons with a distribution similar to that of the whole population. GFR α 1 and GFR α 2 mRNAs were also distributed among neurons of all sizes, although a significant over-representation of intermediate neurons was detected. In contrast to GFR α 1 and GFR α 2, GFR α 3 was present in very few small neurons and not present at all in large neurons. Because there are very few or no GFR α receptor-expressing neurons that do not contain Ret (see below), these results suggest that GFR α 1, GFR α 2 and GFR α 3 are expressed in discrete populations of small neurons whereas they are, to a large extent, coexpressed in the intermediate neurons, thereby leading to an over-representation of this population in the size frequency histogram.

GDNF-null mutant mice display a 23% loss of DRG neurons at birth (Moore et al., 1996). In an effort to determine whether these mice display a loss of a selective subpopulation of DRG neurons, we investigated the cell size distribution of the Ret-positive population of DRG neurons in these mice (Fig. 2C). Interestingly, we found a marked shift towards larger neurons in GDNF-null mutant mice, indicating that there is a

predominant loss of small neurons expressing Ret in newborn GDNF-deficient mice.

GFR α 3 is co-localized with GFR α 1 and GFR α 2, whereas GFR α 1 and GFR α 2 are mostly present in distinct populations of sensory neurons

In situ hybridization was performed in neonatal mice for Ret, GFR α 1, GFR α 2 and GFR α 3 using the probes separate or in combinations, and the percentage of DRG neurons labeled was examined (Fig. 2D). Ret was found in 44% of the neurons, as previously shown (Molliver et al., 1997). GFR α 1 was found in 17%, GFR α 2 in 22% and GFR α 3 in 34% of the neurons. Combining GFR α 1, GFR α 2 or GFR α 3 probes with the Ret probe resulted in approximately 50% of DRG neurons labeled. This indicates that virtually all neurons expressing GFR α receptors also express Ret. Consistent with our culture results, combining the GFR α 1 and GFR α 2 probes led to an additive number of labeled neurons, showing that they are expressed in separate subpopulations of DRG neurons. The number of labeled neurons following hybridization with GFR α 1 and GFR α 3 or GFR α 2 and GFR α 3 probes led to only partial additive numbers of labeled neurons compared with when the probes were used separately. This suggests that most GFR α 3-expressing neurons also express GFR α 1 or GFR α 2.

Collaboration of GDNF family ligands and neurotrophins in the developing DRG

A number of in vitro and in vivo studies have shown that many sensory neuron populations require the collaboration of different neurotrophin ligands for their survival during development (Davies et al., 1993; Buchman and Davies, 1993; Elshamy et al., 1997; reviewed by Snider and Wright, 1996; Molliver et al., 1997). Recent studies also show a collaboration between families of neurotrophic factors (Molliver et al., 1997; Bennett et al., 1998; Fundin et al., 1999). We therefore examined whether the neurotrophins and the GDNF family ligands had overlapping, additional or sequential survival effects on DRG sensory neurons during embryonic and postnatal development. We cultured DRG neurons in the presence of one neurotrophin (NGF, BDNF, NT3 or NT4) and one GDNF family ligand in all combinations. Our results show that while fewer neurons are dependent on NGF for their survival at P7 compared with E12, there is an increasing number of neurons requiring the presence of GDNF, NTN or ART during the same period (Fig. 3A-D). However, the combination of NGF with either one of the GDNF family ligands does not increase the survival above NGF alone. We therefore conclude that embryonic NGF-dependent sensory neurons acquire responsiveness to GDNF family ligands postnatally.

As with the GDNF family ligands, few embryonic sensory neurons survived in presence of brain-derived neurotrophic factor (BDNF). A survival effect of BDNF was seen at birth and further increased at P7. However, when neurons were cultured with BDNF and GDNF family ligands combined, no significant additive effect on the survival of the DRG neurons was detected (Fig. 3B).

In contrast to NGF and BDNF, which support a largely overlapping population of postnatal sensory neurons in the presence of the studied GDNF family ligands, NT3 (Fig. 3C) and NT4 (Fig. 3D) supported different neuronal populations at

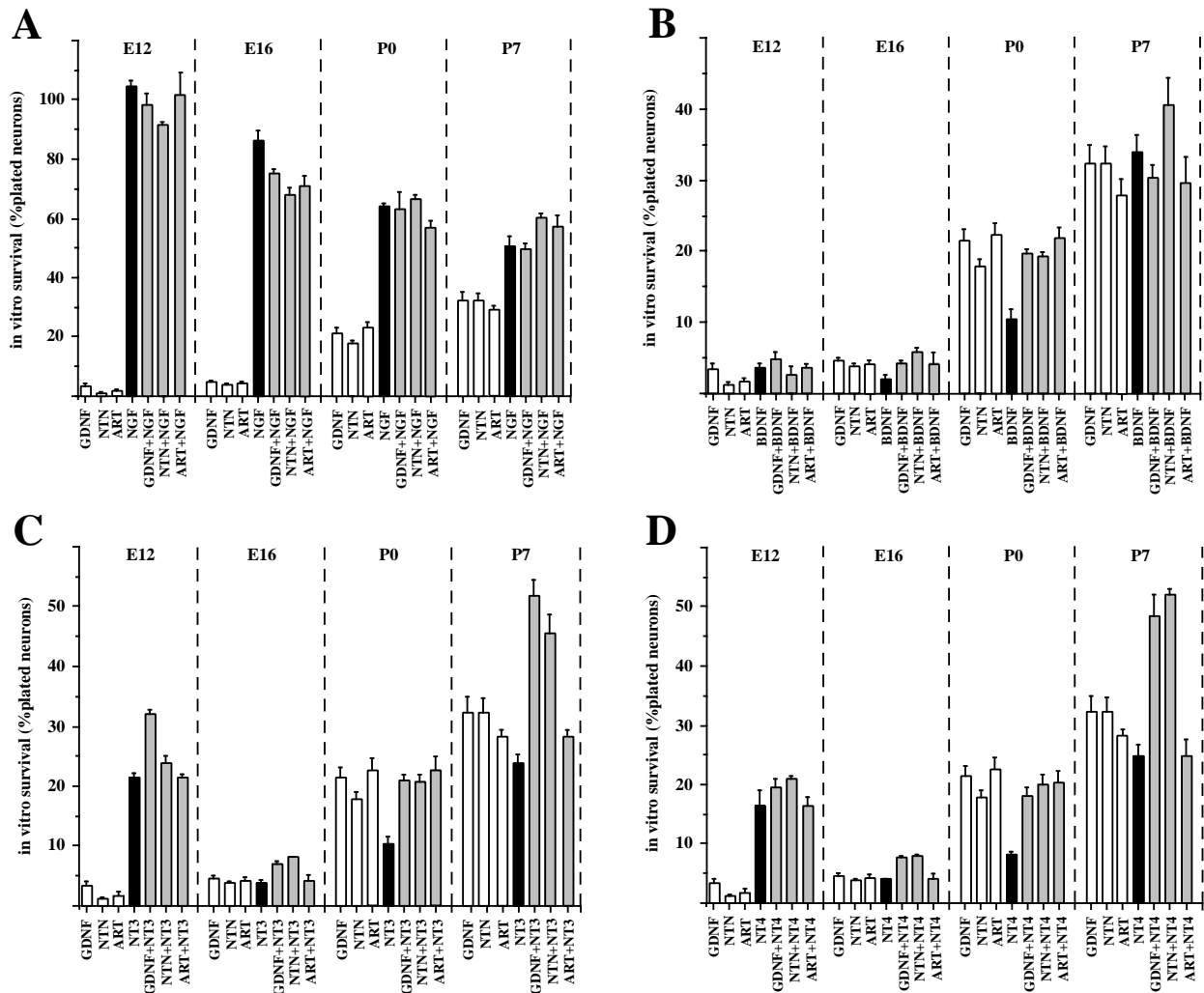


Fig. 3. Effect of the combination of NGF (A), BDNF (B), NT3 (C) or NT4 (D) with GDNF, NTN or ART on survival of developing DRG neurons in primary culture. The survival-promoting effects were studied on E12, E16, P0 and P7 neurons. The cells were incubated for two days in presence of one of the neurotrophins, GDNF, NTN or ART as a control for the cultures maintained in presence of each neurotrophin combined with each one of the GDNF family ligands. NGF, BDNF, NT3, NT4, GDNF and NTN were added at 10 ng/ml, and ART-conditioned medium (as well as control condition medium was diluted at 1/800). Values represent the mean \pm s.e.m. of at least three experiments made in triplicate ($n=9-15$). The white bars show the results for the GDNF family ligands alone, the black bar is the neurotrophin alone and the gray bars represent the survival when the neurotrophin is combined with one of the GDNF family ligand. (A) No additional survival when NGF is combined with the GDNF family ligands. (B) The survival effect of BDNF on postnatal DRG neurons does not add to the late survival effect of GDNF, NTN or ART. NT3 (C) and NT4 (D) rescue more P7 DRG neurons when combined with GDNF or NTN.

P7 from GDNF and NTN, since a marked additive survival was seen when they were combined. However, the survival of distinct populations of P7 sensory neurons emerged during the first postnatal week, because no additional survival was seen in cultured P0 neurons. However, neither NT3 nor NT4 displayed an additional survival effect when combined with ART.

DISCUSSION

GDNF family ligands: survival of DRG neurons and receptor expression

In vitro and expression studies have substantiated a role for GDNF family ligands in mammalian somatosensory neuron

development. However, owing to the limited number of stages analyzed, their effects on neuronal survival during embryonic and postnatal development have not been clearly defined. We find that although sensory neurons express GDNF family receptor mRNAs embryonically, they do not respond to the ligands by survival in culture. The fact that GDNF family ligands do not support neuronal survival during the early periods of target innervation indicates that they may play other functions embryonically. In contrast, we show that the GDNF family ligands play a significant role for neuronal survival postnatally.

Several observations strongly imply a role for GDNF family ligands in terminal innervation and stabilization of the functional nerve endings. Using in situ hybridization, GDNF ligands have been shown to be expressed in the root sheath of the follicle sinus complex whereas GFR α 1 mRNA was

expressed in the terminal Schwann cells and immunoreactivity against the GFR α 1 protein is present throughout the long finger-like processes of these cells. Because of the intimate relation with the axon in the neuromuscular junction, these processes have been suggested to guide the arriving axon to its correct location for the formation of a functional nerve ending (Son and Thompson, 1995a,b). Moreover, Nguyen et al. (1998) showed that an overexpression of GDNF at early postnatal stages leads to a delayed synapse elimination. However, further studies are required in order to address directly the possible role of GDNF family ligands in terminal innervation.

In the adult rat, the cytochemical properties of the Ret-, GFR α 1- and GFR α 2-expressing DRG neurons are largely known. Close to 80% of the Ret mRNA-expressing neurons are IB4-positive, among those, 28% are TrkA-positive (Bennett et al., 1998). IB4-positive neurons represent half of the small neuron population, they are unmyelinated axons principally from nociceptors and thermoceptors, and the other half express neuropeptides and TrkA (Averill et al. 1995; Molliver et al., 1995; Silverman and Kruger, 1990). The remaining Ret-positive/IB4-negative neurons contain neurofilaments, and are myelinated medium and large neurons. Thus, the Ret-positive neurons can be divided in three populations, small neurons expressing IB4; small neurons expressing TrkA and IB4; and myelinated medium and large neurons expressing neurofilament. Interestingly, we report a shift of Ret-expression towards more intermediate neurons in GDNF-deficient animal, indicating a selective loss of small Ret-expressing neurons in the absence of GDNF. We find in the neonatal mouse that GFR α 1, GFR α 2 and GFR α 3 are expressed in the Ret-positive population of DRG neurons. Of these, GFR α 1 and GFR α 2 are expressed in separate subpopulations, whereas GFR α 3 is expressed in populations overlapping with GFR α 1 and GFR α 2. It is interesting to note that in our size frequency histogram of Ret- and GFR α -expressing neurons the majority of the Ret-expressing cells correspond to small neurons, presumably nociceptors, whereas the majority of GFR α 1-, GFR α 2- and GFR α 3-expressing neurons are of significantly larger sizes. Thus, from these data we can infer that the co-expression of GFR α 3/GFR α 2 and GFR α 3/GFR α 1 occurs predominantly in the populations of medium and large DRG neurons.

Considering the binding specificity of the GDNF family ligands with each GFR α (Rosenthal, 1999), our *in vitro* results are consistent with our *in vivo* studies. Indeed, when we show that GFR α 1 and GFR α 2 are mostly expressed on different cells in the DRG, we also find in primary culture of DRG neurons that more cells survive in presence of GDNF and NTN than when only one of the two ligands is added to the medium. Moreover, we report that GFR α 3 is mostly colocalized with GFR α 1 and GFR α 2 in the neonatal DRG and that the addition of ART to a medium containing GDNF or NTN does not increase the percentage of surviving cells when compared with the culture treated with ART, GDNF or NTN alone.

Surprisingly, our results on P15 neurons show that a treatment with ART and GDNF and/or NTN leads to less neuronal survival than either ligands alone, indicating that ART can elicit survival-opposing effects. Several unexpected and unexplained activities of the neurotrophins have also been observed where, instead of acting as survival- and neurite growth-promoting factors, they elicit negative effects on neuronal survival (Ibanez et al., 1993;

Fundin et al., 1997; McAllister et al. 1997). Whereas survival by neurotrophins is elicited by binding to Trk tyrosine kinase receptor, cell death follows the activation of a common p75 neurotrophin receptor (p75^{NTR}, Rodriguez-Tebar et al., 1990, 1992; Ryden et al., 1995) that shows structural similarities to the tumor necrosis factor receptor (p55^{TNFR}) and Fas (Meakin and Shooter, 1992). Also similar to the cytotoxic receptors p55^{TNFR} and Fas, the short cytoplasmic tail of p75^{NTR} contains a death domain (Liepinsh et al., 1997). Similar cell survival-opposing effects within the GDNF family of ligands at later developmental stage (P15) could be of possible biological relevance. A competitive antagonistic action of ART at the GFR α 1 and GFR α 2 receptors seems unlikely considering their preferential affinity for GDNF and NTN. However, if GDNF family ligands can form heterodimers, neuronal loss could be caused by different receptor interactions of heterodimers compared with homodimers. Finally, the possibility that ART activates a yet unidentified receptor with different functions than Ret can not be excluded at present.

Cooperative effects between neurotrophins and GDNF family ligands on DRG neurons

The sequential survival-promoting effects of NGF and GDNF during development, with NGF supporting neurons at earlier stages and GDNF at later stages, combined with the finding that there is no additive survival effect at P7, suggest that embryonic NGF-dependent neurons acquire GDNF responsiveness postnatally. This finding is in agreement with a number of recent studies. Ret expression increases in DRG neurons during development and reaches adult levels at P7 (Molliver et al., 1997). This increase followed by a downregulation of TrkA in many neurons (Molliver et al., 1997; Molliver and Snider, 1997; Bennett et al., 1996, 1998). A switch from early NGF dependency to later GDNF dependency has also been shown in the follicle sinus complex where the reticular and lanceolate endings, which are absent in NGF and TrkA-null mutant mice embryonically (Fundin et al., 1997), are lost postnatally in GDNF-null mutant mice (Fundin et al., 1999). In DRGs, NGF signaling through TrkA supports the survival of peptidergic nociceptive neurons postnatally (Patel et al., 2000). NGF supports both peptidergic and nonpeptidergic nociceptive neurons embryonically. The embryonic NGF-dependent nonpeptidergic neurons acquire GDNF responsiveness postnatally (Molliver et al., 1997; Bennett et al., 1998; Fundin et al., 1997; Stucky and Lewin, 1999). Our results confirm the previous findings and extend the concept by showing that most postnatal NTN- and ART-dependent neurons are also supported embryonically by NGF.

In contrast to NGF, BDNF did not promote the survival of embryonic neurons, but promoted the survival of 35% of P7 DRG neurons. These results are consistent with the studies of BDNF-receptor mutant mice, where 35% of the DRG sensory neurons appear to be lost postnatally (Minichiello et al., 1995). The BDNF receptor, TrkB, is expressed by a subpopulation of intermediate mechanoreceptive neurons (McMahon et al., 1994; Carrol et al., 1998), and we show that all three GFR α s are also mainly expressed in intermediate cells. Moreover, there was no additive effect on the survival of the DRG neurons when combining BDNF with either one of the GDNF family ligands, when compared with the culture in presence of a single factor, indicating that these could overlap with TrkB-positive BDNF-

dependent neurons. Our findings therefore indicate that the subpopulation of neurons maintained by GDNF family ligands overlap with the subpopulation of BDNF-dependent neurons. BDNF has been shown to be essential for normal mechanical sensitivity (but not survival) of low-threshold slowly adaptive mechanoreceptive terminals (Merkel end-organ terminals) that respond to skin indentation (Carroll et al., 1998).

Although, both BDNF and NT4 bind TrkB present on responsive neurons, they activate different intracellular pathways and support distinct subpopulations of sensory neurons (Davies et al., 1993; Erickson et al., 1996; Minichiello et al., 1998; Carrol et al., 1998). NT4, but not BDNF, regulates the survival of a subclass of hair follicle receptors, the low-threshold D-hair afferents that respond dynamically to skin stimulation (Stucky et al., 1998; for a review see Snider and Wright, 1996). Embryonic sensory neurons responded to NT4 transiently at E12, whereas few E16 and neonatal neurons were supported, similar to previous studies (Davies et al., 1993; Paul et al., 1995). Survival by NT4 re-emerged at P7 when 24% of the neurons were maintained. At this stage the effects of NT4 and GDNF or NTN were additive, indicating that most postnatal NT4-dependent and GDNF- and NTN-responding neurons belong to different subpopulations.

NT3 supported 22% of E12 neurons, which lost responsiveness between E12 and E16 as previously shown (Paul et al., 1995). Between E16 and P7, a progressively increased number of neurons survived in presence of the neurotrophin. Most E11 DRG neurons express TrkC, the NT3 receptor. By E13, many of these cells downregulate TrkC and lose their responsiveness to NT3 (Ernfors et al., 1992; Buchman and Davies, 1993). After birth, TrkC is expressed only in the largest DRG neurons, consisting of approximately 20% of the total population (Snider et Wright, 1996). Thus, the NT3-responding cells at E12 in our culture consist of neurons that have not yet downregulated TrkC. By E16 and P0 few neurons expressed TrkC and survived with NT3. However, at P7, NT3 regulated the survival of 23% of the neurons. Late embryonically and postnatally NT3 controls the survival of all trunk proprioceptive neurons (Ernfors et al., 1994) as well as some Merkel cell afferents (Airaksinen et al., 1996; Fundin et al., 1997). NT3 and GDNF/NTN effects on neuronal survival were additive at P7, indicating that the NT3-dependent neurons are distinct from those regulated by GDNF and NTN. Thus, we find that GDNF family ligands may not regulate NT3- or NT4-dependent proprioceptive afferents, slowly adapting mechanoreceptor afferents and D-hair afferents.

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REFERENCES

Airaksinen, M. S., Koltzenburg, M., Lewin, G. R., Masu, Y., Helbig, C., Wolf, E., Brem, G., Toyka, K. V., Thoenen, H. and Meyer, M. (1996). Specific subtypes of cutaneous mechanoreceptors require neurotrophin-3 following peripheral target innervation. *Neuron* **16**, 287-295.

Averill, S., McMahon, S. B., Clary, D. O., Reichardt, L. F. and Priestley, J. V. (1995). Immunocytochemical localization of trkA receptors in chemically identified subgroups of adult rat sensory neurons. *Eur. J. Neurosci.* **7**, 1484-1494.

Baloh, R. H., Tansey, M. G., Golden, J. P., Creedon, D. J., Heuckeroth, R. O., Keck, C. L., Zimonjic, D. B., Popescu, N. C., Johnson, E. M. Jr and Milbrandt, J. (1997). TrnR2, a novel receptor that mediates Neurturin and GDNF signaling through Ret. *Neuron* **18**, 793-802.

Baloh, R. H., Tansey, M. G., Lampe, P. A., Fahrner, T. J., Enomoto, H., Simburger, K. S., Leitner, M. L., Araki, T., Johnson, E. M. Jr and Milbrandt, J. (1998a). Artemin, a novel member of the GDNF ligand family, supports peripheral and central neurons and signals through the GFR α 3-ret receptor complex. *Neuron* **21**, 1291-1302.

Baloh, R. H., Gorodinsky, A., Golden, J. P., Tansey, M. G., Keck, C. L., Popescu, N. C., Johnson, E. M. Jr and Milbrandt, J. (1998b). GFR α 3 is an orphan member of the GDNF/neurturin/persipin receptor family. *Proc. Natl. Acad. Sci. USA* **95**, 5801-5806.

Bennett, D. L. H., Averill, S., Clary, D. O., Priestley, J. V. and McMahon, S. B. (1996). Postnatal changes in the expression of the trkA high affinity NGF receptor in primary sensory neurons. *Eur. J. Neurosci.* **8**, 2204-2208.

Bennett, D. L. H., Michael, G. J., Ramachandran, N., Munson, J. B., Averill, S., Yan, Q., McMahon, S. B. and Priestley, J. V. (1998). A distinct subgroup of small DRG cells express GDNF receptor components and GDNF is protective for these neurons after nerve injury. *J. Neurosci.* **18**, 3059-3072.

Buj-Bello, A., Buchman, V. L., Horton, A., Rosenthal, A. and Davies, A. M. (1995). GDNF is an age-specific survival factor for sensory and autonomic neurons. *Neuron* **15**, 821-828.

Buj-Bello, A., Adu, J., Pinon, L. G., Horton, A., Thompson, J., Rosenthal, A., Chinchetru, M., Buchman, V. L. and Davies, A. M. (1997). Neurturin responsiveness requires a GPI-linked receptor and the Ret receptor tyrosine kinase. *Nature* **387**, 721-724.

Buchman, V. L. and Davies, A. M. (1993). Different neurotrophins are expressed and act in a developmental sequence to promote the survival of embryonic sensory neurons. *Development* **118**, 989-1001.

Cacalano, G., Farinas, I., Wang, L., Hagler, K., Forgie, A., Moore, M., Armanini, M., Phillips, H., Ryan, A. M., Reichardt, L. F., Hynes, M., Davies, A. M. and Rosenthal, A. (1998). GFR α 1 is an essential receptor component for GDNF in the developing nervous system and kidney. *Neuron* **21**, 53-62.

Carr, P. A. and Nagy, J. I. (1993). Emerging relationships between cytochemical properties and sensory modality transmission in primary neurons. *Brain Res. Bull.* **30**, 209-219.

Carroll, P., Lewin, G. R., Koltzenburg, M., Toyka, K. V. and Thoenen, H. (1998). A role for BDNF in mechanosensation. *Nat. Neurosci.* **1**, 42-46.

Creedon, D. J., Tansey, M. G., Baloh, R. H., Osborne, P. A., Lampe, P. A., Fahrner, T. J., Heuckeroth, R. O., Milbrandt, J. and Johnson, E. M., Jr (1997). Neurturin shares receptors and signal transduction pathways with glial cell line-derived neurotrophic factor in sympathetic neurons. *Proc. Natl. Acad. Sci. USA* **94**, 7018-7023.

Davies, A. M., Horton, A., Burton, L. E., Schmeizer, C., Vandlen, R. and Rosenthal, A. (1993). Neurotrophin-4/5 is a mammalian-specific survival factor for distinct populations of sensory neurons. *J. Neurosci.* **13**, 4961-4967.

Durbeck, P., Marcos-Gutierrez, C. V., Kilkenny, C., Grigoriou, M., Wartiovaara, K., Suvanto, P., Smith, D., Ponder, B., Costantini, F., Saarma, M., Sariola, H. and Pachnis, V. (1996). GDNF signalling through the Ret receptor tyrosine kinase. *Nature* **381**, 789-793.

Elshamy, W. M. and Ernfors, P. (1996). A local action of neurotrophin-3 prevents the death of proliferating sensory neuron precursor cells. *Neuron* **16**, 963-972.

Elshamy, W. M. and Ernfors, P. (1997). Brain-derived neurotrophic factor, neurotrophin-3, and neurotrophin-4 complement and cooperate with each other sequentially during visceral neuron development. *J. Neurosci.* **17**, 8667-8675.

Enokido, Y., de Sauvage, F., Hongo, J. A., Ninkina, N., Rosenthal, A., Buchman, V. L. and Davies, A. (1998). GFR α 4 and the tyrosine kinase Ret form a functional receptor complex for persipin. *Curr. Biol.* **8**, 1019-1022.

Enomoto, H., Araki, T., Jackman, A., Heuckeroth, R. O., Snider, W. D., Johnson, E. M. and Milbrandt, J. (1998). GFR α 1-deficient mice have deficits in the enteric nervous system and kidneys. *Neuron* **21**, 317-324.

Erickson, J. T., Conover, J. C., Borday, V., Champagnat, J., Barbacid, M., Yancopoulos, G. and Katz, D. M. (1996). Mice lacking brain-derived neurotrophic factor exhibit visceral sensory neuron losses distinct from mice

- lacking NT4 and display a severe developmental deficit in control of breathing. *J. Neurosci.* **16**, 5361-5371.
- Ernfors, P., Merlio, J. P. and Persson, H.** (1992). Cells expressing mRNA for neurotrophins and their receptors during embryonic rat development. *Eur. J. Neurosci.* **4**, 1140-1158.
- Ernfors, P., Lee, K. F., Kucera, J. and Jaenisch, R.** (1994). Lack of neurotrophin-3 leads to deficiencies in the peripheral nervous system and loss of limb proprioceptive afferents. *Cell* **77**, 503-512.
- Forgie, A., Doxakis, E., Buj-Bello, A., Wyatt, S. and Davies, A. M.** (1999). Differences and developmental changes in the responsiveness of PNS neurons to GDNF and neurturin. *Mol. Cell Neurosci.* **13**, 430-440.
- Fundin, B. T., Silos-Santiago, I., Ernfors, P., Fagan, A. M., Aldskogius, H., DeChiara, T. M., Phillips, H. S., Barbacid, M., Yancopoulos, G. D., Rice, F. L.** (1997). Differential dependency of cutaneous mechanoreceptors on neurotrophins, trk receptors, and p75. *Dev. Biol.* **190**, 94-116.
- Fundin, B. T., Mikaels, A., Westphal, H. and Ernfors, P.** (1999). A rapid and dynamic regulation of GDNF-family ligands and receptor correlate with the developmental dependency of cutaneous sensory innervation. *Development* **126**, 2597-2610.
- Hearn, C. J., Murphy, M. and Newgreen, D.** (1998). GDNF and ET-3 differentially modulate the numbers of avian enteric neural crest cells and enteric neurons in vitro. *Dev. Biol.* **197**, 93-105.
- Heuckeroth, R. O., Lampe, P. A., Jonhson, E. M. and Milbrandt, J.** (1998). Neurturin and GDNF promote proliferation and survival of enteric neuron and glial progenitors in vitro. *Dev. Biol.* **200**, 116-119.
- Heuckeroth, R. O., Enomoto, H., Grider, J. R., Golden, J. P., Hanke, J. A., Jackman, A., Molliver, D. C., Bardgett, M. E., Snider, W. D., Johnson, E. M., Jr and Milbrandt, J.** (1999). Gene targeting reveals a critical role for Neurturin in the development and maintenance of enteric, sensory, and parasympathetic neurons. *Neuron* **22**, 253-263.
- Horger, B. A., Nishimura, M. C., Armanini, M. P., Wang, L. C., Poulsen, K. T., Rosenblad, C., Kirik, D., Moffat, B., Simmons, L., Jonhson, Jr E., Milbrandt, J., Rosenthal, A., Bjorklund, A., Vandlen, R. A., Hymes, M. A. and Phillips, H.** (1998). Neurturin exerts potent actions on survival and function of midbrain dopaminergic neurons. *J. Neurosci.* **18**, 4929-4937.
- Ibanez, C. F., Ernfors, P., Timmusk, T., Ip, N. Y., Arenas, E., Yancopoulos, G. D. and Persson, H.** (1993). Neurtrophin-4 is a target-derived neurotrophic factor for neurons of the trigeminal ganglion. *Development* **117**, 1345-1353.
- Iwamoto, T., Taniguchi, M., Asai, N., Ohkusu, K., Nakashima, I. and Takahashi, M.** (1993). cDNA cloning of mouse ret proto-oncogene and its sequence similarity to the cadherin superfamily. *Oncogene* **8**, 1087-1091.
- Jing, S., Wen, D., Yu, Y., Hoist, P. L., Luo, Y., Fang, M., Tamir, R., Antonio, L., Hu, Z., Cupples, R., Louis, J. C., Hu, S., Altmock, B. W. and Fox, G. M.** (1996). GDNF-induced activation of the ret protein tyrosine kinase is mediated by GDNF α , a novel receptor for GDNF. *Cell* **86**, 1-20.
- Jing, S., Yu, Y., Hoist, P. L., Fang, M., Tamir, R., Hu, Z., Holst, P. L., Boone, T., Delaney, J., Schultz, H., Zhou, R. and Fox, G. M.** (1997). GFR α 2 and GFR α 3 are two new receptors for ligands of the GDNF family. *J. Biol. Chem.* **272**, 33111-33117.
- Klein, R. D., Sherman, D., Ho, W. H., Stone, D., Bennett, G. L., Moffat, B., Vandlen, R., Simmons, L., Gu, Q., Hongo, J. A., Devaux, B., Poulsen, K., Armanini, M., Nozaki, C., Asai, N., Goddard, A., Phillips, H., Henderson, C. E., Takahashi, M. and Rosenthal, A.** (1997). A GPI-linked protein that interact with Ret to form a candidate neurturin receptor. *Nature* **387**, 717-721.
- Kotzbauer, P. T., Lampe, P. A., Heuckeroth, R. O., Golden, J. P., Creedon, D. J., Johnson, E. M., Jr and Milbrandt, J.** (1996). Neurturin, a relative of glial-cell-line-derived neurotrophic factor. *Nature* **384**, 467-470.
- Lewin, G. R. and Barde, Y. A.** (1996). Physiology of the neurotrophins. *Annu. Rev. Neurosci.* **19**, 289-317.
- Liepinsh, E., Ilag, L. L., Otting, G. and Ibanez, C. F.** (1997). NMR structure of the death domain of the p75 neurotrophin receptor. *EMBO J.* **16**, 4999-5005.
- Lin, L. F., Doherty, D., Lile, J., Bektesh, S. and Collins, F.** (1993). GDNF: a glial cell line-derived neurotrophic factor for midbrain dopaminergic neurons. *Science* **260**, 1130-1132.
- Matheson, C. R., Carnahan, J., Urich, J. L., Bocangel, D., Zhang, T. G. and Yan, Q.** (1997). Glial cell line-derived neurotrophic factor (GDNF) is a neurotrophic factor for sensory neurons: comparisons with the effects of neurotrophins. *J. Neurobiol.* **32**, 22-32.
- McAllister, A. K., Katz, L. C. and Lo, D. C.** (1997). Opposing roles for endogenous BDNF and NT-3 in regulating cortical dendritic growth. *Neuron* **18**, 767-778.
- McMahon, S. B., Armanini, M. P., Ling, L. H. and Phillips, H. S.** (1994). Expression and coexpression of trk receptors in subpopulations of adult primary sensory neurons projecting to identified peripheral targets. *Neuron* **12**, 1161-1171.
- Meakin, S. O. and Shooter, E. M.** (1992). The nerve growth factor family of receptors. *Trends Neurosci.* **15**, 323-331.
- Milbrandt, J., de Sauvage, F. J., Fahrner, T. J., Baloh, R. H., Leitner, M. L., Tansey, M. G., Lampe, P. A., Heuckeroth, R. O., Kotzbauer, P. T., Simburger, K. S. et al.** (1998). Persephin, a novel neurotrophic factor related to GDNF and Neurturin. *Neuron* **20**, 1-20.
- Minichiello, L., Piehl, F., Vazquez, E., Schimmang, T., Hokfelt, T., Represa, J. and Klein, R.** (1995). Differential effects of combined Trk receptor mutations on dorsal root ganglion and inner ear sensory neurons. *Development* **121**, 4067-4075.
- Minichiello, L., Casagrande, F., Tatche, R. S., Stucky, C. L., Postigo, A., Doster, K., Wen, D., Yan, Q. and Klein, R.** (1998). Point mutation in trkB causes loss of NT4-dependent neurons without major effects on diverse BDNF responses. *Neuron* **21**, 335-345.
- Molliver, D. C., Radeke, M. J., Feinstein, S. C. and Snider, W. D.** (1995). Presence or absence of TrkA protein distinguishes subsets of small sensory neurons with unique cytochemical characteristics and dorsal horn projections. *J. Comp. Neurol.* **361**, 404-416.
- Molliver, D. C., Wright, D. E., Leitner, M. L., Parasadani, A. S. H., Doster, K., Wen, D., Yan, Q. and Snider, W. D.** (1997). IB4-binding DRG neurons switch from NGF to GDNF dependence in early postnatal life. *Neuron* **19**, 849-861.
- Molliver, D. C. and Snider, W. D.** (1997). Nerve growth factor receptor TrkA is down-regulated during postnatal development by a subset of dorsal root ganglion neuron. *J. Comp. Neurol.* **381**, 428-38.
- Moore, M. W., Klein, R. D., Farinas, I., Sauer, H., Armanini, M., Phillips, H., Reichardt, L. F., Ryan, A. M., Carver-Moore, K. and Rosenthal, A.** (1996). Ret and neuronal abnormalities in mice lacking GDNF. *Nature* **382**, 76-79.
- Naveilhan, P., Elshamy, W. M. and Ernfors, P.** (1997). Differential regulation of mRNAs for GDNF and its receptors Ret and GDNFR alpha after sciatic nerve lesion in the mouse. *Eur. J. Neurosci.* **9**, 1450-1460.
- Naveilhan, P., Baudet, C., Mikaels, A., Shen, L., Westphal, H. and Ernfors, P.** (1998). Expression and regulation of GFR α 3, a glial cell line-derived neurotrophic factor family receptor. *Proc. Natl. Acad. Sci. USA* **95**, 1295-1300.
- Nguyen, Q. T., Parsadani, A. S., Snider, W. D. and Lichtman, J. W.** (1998). Hyperinnervation of neuromuscular junctions caused by GDNF overexpression in muscle. *Science* **279**, 1725-1729.
- Nishino, J., Moshida, K., Ohfuji, Y., Shimazaki, T., Meno, C., Ohishi, S., Matsuda, Y., Fujii, H., Saijoh, Y. and Hamada, H.** (1999). GFR α 3, a component of the artemin receptor, is required for migration and survival of the superior cervical ganglion. *Neuron* **23**, 725-736.
- Patel, T. D., Jackman, A., Rice, F. L., Kucera, J. and Snider, W. D.** (2000). Development of sensory neurons in the absence of NGF/trkA signaling in vivo. *Neuron* **25**, 345-357.
- Paul, G. and Davies, A. M.** (1995). Trigeminal sensory neurons require extrinsic signals to switch neurotrophin dependence during the early stages of target field innervation. *Dev. Biol.* **171**, 590-605.
- Pichel, J. G., Shen, L., Sheng, H.-Z., Granholm, A. C., Drago, J., Grinberg, A., Lee, E. J., Huang, S.-P., Saarma, M., Hoffer, B. J., Sariola, H. and Westphal, H.** (1996). Defects in enteric innervation and kidney development in mice lacking GDNF. *Nature* **382**, 73-75.
- Rodriguez-Tebar, A., Dechant, G. and Barde, Y. A.** (1990). Binding of brain-derived neurotrophic factor to the nerve growth factor receptor. *Neuron* **4**, 487-492.
- Rodriguez-Tebar, A., Dechant, G., Gotz, R. and Barde, Y. A.** (1992). Binding of neurotrophin-3 to its neuronal receptors and interactions with nerve growth factor and brain-derived neurotrophic factor. *EMBO J.* **11**, 917-922.
- Rosenblad, C., Gronborg, M., Hansen, C., Blom, N., Meyer, M., Johansen, J., Dago, L., Kirik, D., Patel, U. A., Lundberg, C., Trono, D., Bjorklund, A. and Johansen, T. E.** (2000). In vivo protection of nigral dopamine neurons by lentiviral gene transfer of novel GDNF-family member Neublastin/Artemin. *Mol. Cell Neurosci.* **15**, 199-214.
- Rosenthal, A.** (1999). The GDNF protein family: gene ablation studies reveal what they really do and how. *Neuron* **22**, 201-207.
- Rossi, J., Luukko, K., Poteryaev, D., Laurikainen, A., Sun, Y. F., Laakso, T., Eerikainen, S., Tuominen, R., Lakso, M., Rauvala, H., Arumäe, U., Pasternack, M., Saarma, M. and Airaksinen, M. S.** (1999). Retarded

- growth and deficits in the enteric and parasympathetic nervous system in mice lacking GFR α 2, a functional neurturin receptor. *Neuron* **22**, 243-252.
- Ryden, M., Murray-Rust, J., Glass, D., Ilag, L. L., Trupp, M., Yancopoulos, G. D., McDonald, N. Q. and Ibanez, C. F.** (1995). Functional analysis of mutant neurotrophins deficient in low-affinity binding reveals a role for p75LNGFR in NT-4 signalling. *EMBO J.* **14**, 1979-1990.
- Sanchez, M. P., Silos-Santiago, I., Frisen, J., He, B., Lira, S. A. and Barbacid, M.** (1996). Renal agenesis and the absence of enteric neurons in mice lacking GDNF. *Nature* **382**, 70-73.
- Sanicola, M., Hession, C., Worley, D., Carmillo, P., Ehrenfels, C., Walus, L., Robinson, S., Jaworski, G., Wei, H., Tizard, R., Whitty, A., Pepinsky, R. B. and Cate, R. L.** (1997). Glial cell line-derived neurotrophic factor dependent Ret activation can be mediated by two different cell-surface accessory proteins. *Proc. Natl. Acad. Sci. USA* **94**, 6238-6243.
- Silverman, J. D. and Kruger, L.** (1990). Selective neuronal glycoconjugate expression in sensory and autonomic ganglia: relation of lectin reactivity to peptides and enzyme markers. *J. Neurocytol.* **19**, 789-801.
- Snider, W. D. and Wright, D. E.** (1996) Neurotrophins cause new sensation. *Neuron* **16**, 229-232.
- Son, Y. J. and Thompson, W. J.** (1995a). Nerve sprouting in muscle is induced and guided by processes extended by Schwann cells. *Neuron* **14**, 133-141.
- Son, Y. J. and Thompson, W. J.** (1995b). Schwann processes guide regeneration of peripheral axons. *Neuron* **14**, 125-132.
- Stucky, C. L., DeChiara, T., Lindsay, R. M., Yancopoulos, G. D. and Koltzenburg, M.** (1998). Neurotrophin 4 is required for the survival of a subclass of hair follicle receptors. *J. Neurosci.* **18**, 7040-7046.
- Stucky, C. L. and Lewin, G. R.** (1999). Isolectin B4-positive and -negative nociceptors are functionally distinct. *J. Neurosci.* **19**, 6497-505.
- Treanor, J. J. S., Goodman, L., de Sauvage, F., Stone, D. M., Poulsen, K. T., Beck, C. D. and al.** (1996). Characterization of a multicomponent receptor for GDNF. *Nature* **382**, 80-83.
- Trupp, M., Arenas, E., Fainzilber, M., Nilsson, S., Sieber, B. A., Grigoriou, M., Kilkenny, C., Salazar-Gruesso, E., Pachnis, V., Arumäe, U. et al.** (1996). Functional receptor for GDNF encoded by the c-ret proto-oncogene. *Nature* **381**, 785-789.
- Widenfalk, J., Tomac, A., Lindqvist, E., Hoffer, B. and Olson, L.** (1998). GFR α 3, a protein related to GFR α 1, is expressed in developing peripheral neurons and ensheathing cells. *Eur. J. Neurosci.* **10**, 1508-1517.
- Worby, C. A., Vega, Q. C., Chao, H. H. J., Seasholtz, A. F., Thompson, R. C. and Dixon, J. E.** (1998). Identification and characterization of GFR α 3, a novel co-receptor belonging to the glial cell line-derived neurotrophic receptor family. *J. Biol. Chem.* **273**, 3502-3508.