

GDNF and neurturin are target-derived factors essential for cranial parasympathetic neuron development

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SUMMARY

During development, parasympathetic ciliary ganglion neurons arise from the neural crest and establish synaptic contacts on smooth and striate muscle in the eye. The factors that promote the ciliary ganglion pioneer axons to grow toward their targets have yet to be determined. Here, we show that glial cell line-derived neurotrophic factor (GDNF) and neurturin (NRTN) constitute target-derived factors for developing ciliary ganglion neurons. Both GDNF and NRTN are secreted from eye muscle located in the target and trajectory pathway of ciliary ganglion pioneer axons during the period of target innervation. After this period, however, the synthesis of GDNF declines markedly, while that of NRTN is maintained throughout

the cell death period. Furthermore, both *in vitro* and *in vivo* function-blocking of GDNF at early embryonic ages almost entirely suppresses ciliary axon outgrowth. These results demonstrate that target-derived GDNF is necessary for ciliary ganglion neurons to innervate ciliary muscle in the eye. Since the down-regulation of GDNF in the eye is accompanied by down-regulation of GFR α 1 and Ret, but not of GFR α 2, in innervating ciliary ganglion neurons, the results also suggest that target-derived GDNF regulates the expression of its high-affinity coreceptors.

Key words: GDNF, Neurturin, CNTF, GFR α , Ret, Ciliary ganglion, Axon, Chicken

INTRODUCTION

During development, parasympathetic ciliary ganglion neurons arise from the mesencephalic neural crest and establish direct synaptic contacts on ciliary muscle and smooth muscle in the choroid layer of the eye. The factors that promote pioneer axons to grow from the ciliary ganglion towards their target structures in the eye have yet to be identified. Proteins present in chick embryonic eye extracts have long been known to promote the survival of developing ciliary ganglion neurons *in vitro* (Adler et al., 1979; Nishi and Berg, 1981). However, none of the characterized neurotrophins was able to promote the survival of the ciliary ganglion neurons. Ciliary neurotrophic factor (CNTF) has subsequently been isolated and found to have potent survival-promoting effects on the ciliary ganglion neurons *in vitro* (Lin et al., 1989). This discovery led to the assumption that this peptide cytokine plays a major role in parasympathetic neuron development, as was evident from its name. A critical role of CNTF in nervous system development, however, was not substantiated. The expression of CNTF was found to be very low or undetectable in mice during prenatal developmental stages (Sendtner et al., 1994) and mammalian CNTF was poorly secreted from transfected cell lines (Lin et

al., 1989; Sendtner et al., 1990). In addition, no significant phenotype was observed in mice or humans with a null mutation of the CNTF gene (Masu et al., 1993; Takahashi et al., 1994). Furthermore, the highest level of CNTF was detected in myelinating Schwann cells (Rende et al., 1992), suggesting that CNTF is supplied to neurons in a paracrine manner. Since CNTF levels increase markedly after injury in both central and peripheral nervous systems, the primary role of this factor has been considered to be in the regulation of post-injury processes (Sendtner et al., 1990; Sendtner et al., 1992; Winter et al., 1995).

Recent evidence has pointed to another class of neurotrophic factors that is required for the normal development of several parasympathetic neuron populations. Mice carrying a null mutant allele for neurturin (NRTN; Kotzbauer et al., 1996) or its high-affinity receptor, GFR α 2, displayed a similar phenotype, showing a substantial reduction in parasympathetic innervations to the lacrimal and salivary glands (Heuckeroth et al., 1999; Rossi et al., 1999). In addition, NRTN as well as glial cell line-derived neurotrophic factor (GDNF; Lin et al., 1993) has been shown to promote the survival of developing chick ciliary ganglion neurons *in vitro* (Buj-Bello et al., 1995; Hashino et al., 1999b). On the basis of these lines of evidence,

we hypothesized that one or more members of the GDNF family ligands (GDNF, NRTN, Artemin (ARTN; Baloh et al., 1998), and Persephin (PSPN; Milbrandt et al., 1998)) are secreted from target tissues of ciliary ganglion neurons during innervation and that these proteins provide a long-distance cue for growing ciliary ganglion axons. To test this hypothesis, we analyzed proteins synthesized in target eye tissues and determined their identity. In addition, functional biological assays were conducted to assess diffusible components of eye proteins.

MATERIALS AND METHODS

Organotypic culture

Ciliary ganglia were removed from embryonic day 9 (E9) and E16 chick embryos under a dissection microscope. One ganglion was placed in each well of a 24-well culture dishes coated with 0.02% poly-D-lysine (Becton Dickinson, Bedford, MA). The basal medium was serum-free DMEM/F12 (Life Technologies, Grand Island, NY) supplemented with 10 mM Hepes, 25 U/ml penicillin, 1% ITS + PREMIX (Becton Dickinson) and one of the following: recombinant human GDNF, NRTN, ARTN, PSPN, CNTF or BDNF (50 ng/ml each, BDNF, GDNF and CNTF: PeptoTech, Rocky Hill, NJ). Recombinant human NRTN, PSPN and ARTN were prepared as previously described (Creedon et al., 1997; Baloh et al., 1998). The ganglia were incubated with 5% CO₂ at 37°C for 3 days. At the end of the incubation, the explants were fixed with 4% paraformaldehyde for 30 minutes and their neurite processes were visualized with anti-TUJ1 antibody (1:200, Covance Research Products, Richmond, CA). The magnitude of neurite outgrowth was quantitatively evaluated and described as an average of the radial distance of neurite tips from the explant (Hashino et al., 1999b).

Conditioned medium and function-blocking experiments

Eye-conditioned medium was prepared by modifications of the previously described procedure (Hashino et al., 1999a). Briefly, eyes with attached connective tissues were removed from E5 or E12 chick embryos. Ten E5 eyes or two E12 eyes were placed in a well of 24-well culture dishes that contained 800 µl of serum-free medium. The eyes were incubated for 2 days at 37°C and the culture medium was collected and filtered through a low protein-binding PVDF membrane filter. The conditioned medium was diluted 1:1 in defined DMEM/F12 culture medium. Function-blocking antibodies to GDNF (R&D Systems) or NRTN (PeptoTech, Rocky Hill, NJ) at 0.1, 0.2 or 0.5 µg/ml were added to either eye-conditioned medium or defined culture medium containing GDNF, NRTN or CNTF (10 ng/ml, each) prior to the start of an incubation period. E9 ciliary ganglion explants were cultured for 48 hours, after which the mean neurite outgrowth was measured as described above.

Dissociated neuron culture

Ciliary ganglion neurons from E9 or E16 chick embryos were dissociated as previously described (Hashino et al., 1999b). Dissociated neurons were plated on 24-well tissue culture dishes coated with poly-D-lysine (200 µg/ml) and laminin (50 µg/ml, Gibco), and incubated in serum-free medium supplemented with GDNF, NRTN, ARTN, PSPN, CNTF, BDNF (50 ng/ml, each), or no factor (control). Neuronal survival was determined 2 days after the start of incubation by counting phase-bright cells with a neuronal morphology (Hashino et al., 1999b).

Western blot analysis and production of an anti-GFRα2 antibody

Freshly dissected chick embryonic ciliary ganglia or eyes were frozen on dry ice and kept at -80°C until processed. Approximately 120 (E6),

60 (E9), 30 (E12) and 20 (E16) ciliary ganglia were collected to equalize the volume of tissues from different embryonic stages. Tissues were homogenized by several passages through a 23-gauge syringe needle in boiling lysis buffer (10 mM Tris, pH 7.4, 1% SDS, 1 mM Na₃VO₄). Samples were centrifuged at 14,000 *g* for 5 minutes at 4°C, the resulting supernatant was added to an equal volume of sample buffer (100 mM Tris, pH 6.8, 4% SDS, 0.2% Bromophenol Blue, 2% β-mercaptoethanol (for eye lysates only) and 20% glycerol). The protein concentration was determined using the DC protein assay kit (Bio-Rad, Hercules, CA). In each lane, equal amount of protein was run on a 7.5%, 10% or 12% SDS-polyacrylamide gel and transferred to a PVDF membrane (Bio-Rad). The blots were incubated sequentially in blocking buffer (Tropix, Bedford, MA) supplemented with 0.1% Tween 20 overnight at 4°C, a primary antibody overnight at 4°C and alkaline phosphatase-conjugated secondary antibody (Jackson Immuno Research, West Grove, PA). Primary antibodies used in this study were anti-Ret (1:300, Santa Cruz Biotechnology, Santa Cruz, CA), anti-GFRα1 (1:3000, Transduction Laboratories, Lexington, KY), anti-CNTFRα (1:1000, von Holst et al., 1997), anti-gp130 (1:500, Santa Cruz Biotechnology), anti-NRTN (1:1000, PeptoTech), anti-GDNF (1:500, Santa Cruz Biotechnology). A polyclonal GFRα2 antibody was produced by immunizing rabbits with a synthetic peptide corresponding to an extracellular domain of chicken GFRα2 (aa 375-389: NH₂-KVEKSPALPDDINDS-CONH₂). The resultant antiserum was purified with immobilized protein G. This antibody was used for western blot analysis at the concentration of 0.4-0.8 µg/ml. The blots were visualized with the Western-Star chemiluminescent detection system (Tropix) and exposed to X-ray film.

Quantitative RT-PCR

The expression of GFRα1, GFRα2, Ret and L27 mRNAs in E5 and E15 ciliary ganglion neurons were analyzed by real-time RT-PCR. Poly(A)⁺ RNA was isolated from freshly dissected ciliary ganglia using the Oligotex Direct mRNA purification kit (QIAGEN Inc., Valencia, CA) according to the manufacture's instructions. Single-stranded cDNA was then synthesized using the Omniscript reverse transcriptase (QIAGEN Inc.) and Oligo(dT) primers. The resultant cDNA was amplified on the ABI PRISM 7700 Sequence Detection System (PE Applied Biosystems, Foster City, CA) using TaqMan Universal PCR Master Mix (PE Applied Biosystems) that contains dNTPs with dUTP, AmpliTaq Gold DNA polymerase, AmpErase UNG, optimized buffer and the passive reference dye. For each PCR reaction, a mixture containing cDNA template (5 ng), Master Mix (1×), forward and reverse primers (400 nM) and TaqMan probe (200 nM) was placed in a 96-well MicroAmp Optical Reaction Plate with Optical Caps (PE Applied Biosystems). In most cases, the PCR reaction was run under the following conditions: 1×, 50°C, 2 minutes; 1×, 95°C, 10 minutes; 55×, 95°C, 15 seconds, 60°C, 1 minute; 1×, 4°C, hold. After amplification, the PCR products were analyzed with the ABI PRISM sequence detection software. To check if amplification yields PCR products of a single molecular mass, the PCR products were electrophoresed on 2.5% agarose gels containing ethidium bromide. To check for DNA contamination, purified mRNA was run for the PCR reaction. To check the linearity of the detection system, a cDNA dilution series (1, 1:10, 1:100, 1:1000) was amplified with a primer pairs and TaqMan probe and a correlation coefficient was calculated from the standard curve that displays threshold cycles (C_T) as a function of log₁₀ cDNA concentrations. The mRNA level for each probe (x) relative to L27 mRNA (internal control) was calculated and described as follows: mRNA(x)=2^{C_T(L27)-C_T(x)}×100. The primer sets and TaqMan probes used were: GFRα1 [forward, 5'-AAC TCT GTC TTA ATG AGA ATG CTA TTG G-3', reverse, 5'-GAA TGA GTG TGC TTG GAT AGA-3' and probe, 5'FAM (6-carboxy fluorescein)-TCT CCA CCA GCC ACA TAT CCT CGG A-3'TAMRA (6-carboxy-tetramethylrhodamine)], GFRα2 [forward, 5'-CCC TCA CCT CCC ATC ACA AT-3', reverse, 5'-TTC TGT CCG

TGC TCC TGG AT-3' and probe, 5'FAM-CAA GGT GGA GAA AAG TCC TGC CTT GCC-3'TAMRA], Ret [forward, 5'-GCC AAG AGA GAG GAA CTC AAC-3', reverse, 5'-AAA TTC TGT GTC GTT CAC CAA-3' and probe, 5' FAM-CAG TCA CAG AGC ATC GTT CCT TCC A-3'TAMRA] and L27 [forward, 5'-AAG GCT GTC ATC GTG AAG AAC AT-3', reverse, 5'-TCG ATG CCT GCC ACC AA-3' and probe, 5'FAM-CTG ATC GGC CCT ACA GCC ACG C-3'TAMRA].

Immunohistochemistry

E7 chick embryos were fixed in 4% paraformaldehyde for 4 hours and embedded in paraffin. Transverse sections (10 μ m) of the head were incubated with blocking solution [0.3% Triton X-100, 5% BSA, 10% normal horse serum in hi-salt (1.8% NaCl) PBS], and then either with anti-GDNF (1:100, Santa Cruz Biotechnology), anti-NRTN (1:100, PeproTech) or anti-Ret (1:100, Santa Cruz Biotechnology) in blocking solution at 4°C overnight. Thereafter, the sections were incubated with biotinylated secondary antibody (Vector Lab, 5 μ l/ml) and the reaction product was visualized by the biotin-avidin-HRP detection system with the Vector VIP substrate kit. To confirm the specificity of the staining, sections were processed with omission of incubation with a primary antibody.

In vivo axon collapse assay

The function-blocking GDNF antibody (200 μ g/ml in PBS, 150 μ l) or PBS (150 μ l) was injected into the chorioallantoic membrane through a window cut in the shell of E3.5 embryos. The eggs were incubated for a further 4 days, harvested and fixed in 4% paraformaldehyde. After dissecting an eye with an attached ciliary ganglion, crystals of DiI (Molecular Probes, Eugene, OR) were implanted into the ciliary ganglion and the tissue was incubated at 37°C for 3-5 days. At the end of the incubation, the frontal part of the eye as well as the lens was removed, thereby flattening the lateral-posterior surface of the eye. DiI-labeled processes emerging from the ciliary ganglion were examined using a fluorescence microscope. Some of the embryos grown in the presence of the GDNF antibody were fixed and embedded in paraffin. Transverse serial sections of the whole heads were collected and stained with Toluidine Blue.

RESULTS

GDNF/NRTN and CNTF exert neurotrophic effects on ciliary ganglion neurons in a distinctive manner

To identify factors that have trophic effects on developing ciliary ganglion neurons, we first evaluated the neurite outgrowth-promoting effects of the GDNF family ligands using organotypic culture. GDNF and NRTN promoted extensive neurite outgrowth from E9 ciliary ganglia, whereas ARTN had much less effect and PSPN had no effect (Fig. 1). The positive effects of GDNF and NRTN were comparable to those of CNTF. GDNF, NRTN and CNTF also promoted the survival of approximately 90% of E9 dissociated ciliary ganglion neurons, which was in contrast to 5% survival of the same neuron population cultured with PSPN, BDNF or without a neurotrophic factor. Approximately 20% of the ciliary ganglion neurons survived for 2 days in the presence of ARTN at 50 ng/ml.

The strong neurotrophic effects of GDNF and NRTN seen at E9 were not observed for E16 ciliary ganglion neurons. Unlike E9 ciliary ganglion neurons, a subpopulation of E16 ciliary ganglion neurons survived and extended neurite processes in the absence of an exogenous factor. Neither GDNF nor NRTN promoted a significant outgrowth from E16 ciliary ganglion neurons compared to controls, whereas CNTF had positive effects on the E16 ciliary ganglion neuron outgrowth (Fig. 1). Interestingly, however, both GDNF and NRTN promoted the survival of E16 ciliary ganglion neurons

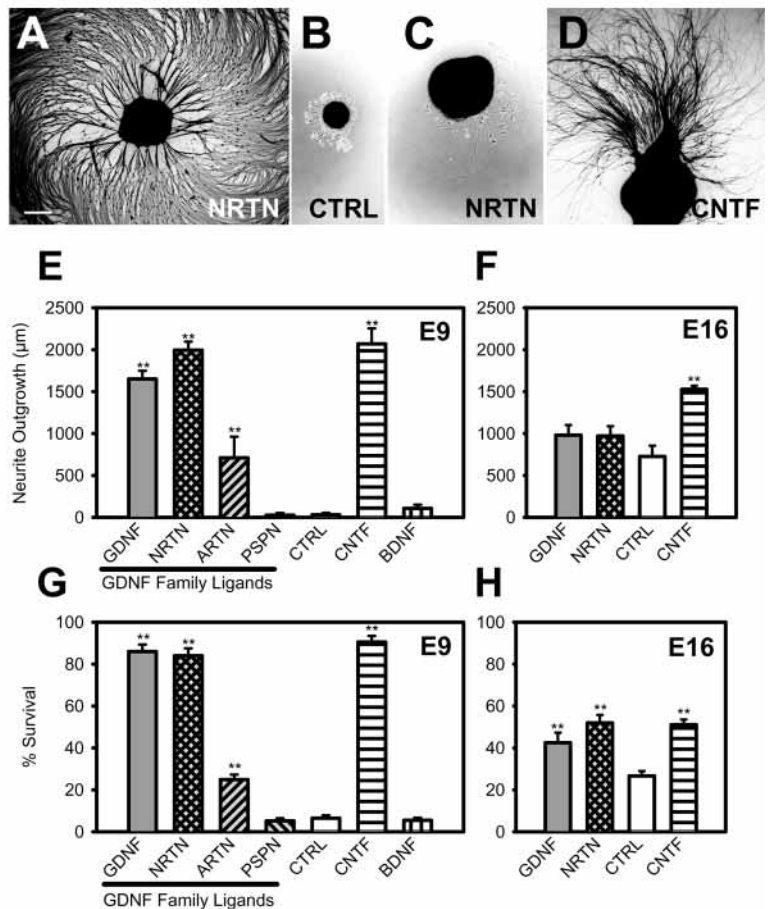
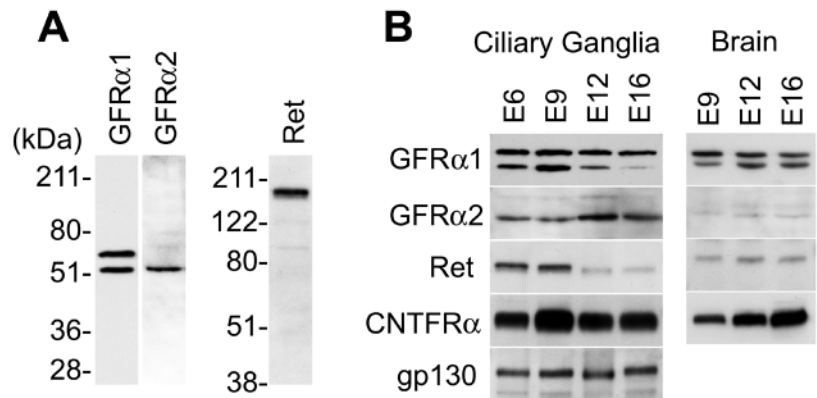


Fig. 1. GDNF and NRTN promote the neurite outgrowth and survival of ciliary ganglion neurons in vitro in a stage-specific manner. (A,B) E9 ciliary ganglion explant with (A) or without (B) NRTN at 50 ng/ml. (C,D) E16 ciliary ganglion explant with NRTN (C) or CNTF (D) at 50 ng/ml. Note that NRTN induces an extensive outgrowth from E9, but not from E16 ciliary ganglia. In contrast, CNTF has positive effects on E16 ciliary ganglion neurite outgrowth. A and D were immunostained with a TUJ1 antibody and B and C were unstained phase-contrast micrographs. Scar bar, 500 μ m. (E,F) Quantitative analysis of neurite outgrowth from E9 (E) or E16 (F) ciliary ganglion explants in the presence of GDNF family ligands, CNTF or BDNF. Graphs show the average neurite length (\pm s.e.) for ciliary ganglion explants grown in medium alone or medium containing either GDNF, NRTN, ARTN, PSPN, CNTF or BDNF (50 ng/ml, for each factor) for 3 days. (G,H) Survival of E9 (G) or E16 (H) ciliary ganglion neurons in the presence of GDNF family ligands, CNTF or BDNF (50 ng/ml for each factor). Results are expressed as a percentage of the neurons that survived after 48 hours in culture (\pm s.e.). Note that the majority of E9 ciliary ganglion neurons are dependent on GDNF or NRTN (G), whereas only a sub-population of E16 ciliary ganglion neurons are supported by these factors (H). Each bar represents data from 7-14 explants. **, Significantly different from the control group ($P < 0.01$).

Fig. 2. Expression of GFR α 1 and Ret proteins declines in ciliary ganglion neurons after target innervation. (A) Western blot characterization of anti-GFR α 1, GFR α 2 and Ret antibodies used in the present study. The GFR α 2 antiserum generated in this study recognizes an approx. 55 kDa protein, equivalent to the estimated size of GFR α 2, in E9 ciliary ganglion neurons. The anti-GFR α 1 antibody recognizes two protein species of approx. 55 and 65 kDa, whereas the anti-Ret antibody recognizes a single protein of 170 kDa in ciliary ganglion neurons. (B) Western blot analysis for GFR α 1, GFR α 2, Ret, CNTFR α and gp130 in ciliary ganglion lysates (left) or whole brain lysates (right) at different embryonic ages.



at a level (40%) significantly higher than the control level (20%).

GFR α 1 and Ret are down-regulated, whereas GFR α 2, CNTFR α and gp130 levels are constant in ciliary ganglion neurons during development

To test if the differences in the stages at which GDNF/NRTN and CNTF exert positive effects on the ciliary ganglion neurons are attributable to differences in their receptor expression, we evaluated the expression level of GDNF/NRTN receptors and CNTF receptors at different embryonic stages by western blot analysis. We first characterized an anti-chicken GFR α 2 antiserum that we generated. Western blot analysis with E9 ciliary ganglion lysates indicated that this antiserum specifically recognized a protein species that migrates at an apparent molecular mass of 55 kDa (Fig. 2A). The size of the recognized protein is consistent with a predicted molecular mass for human GFR α 2 (approx. 55 kDa). In addition, the 55 kDa immunoreactive band was undetectable when primary antibody was preincubated with antigen peptide (data not shown), verifying the specificity of the GFR α 2 antibody. The cross-reactivity of the anti-GFR α 1 and anti-Ret antibodies used in this study were also tested by western blot analysis. These antibodies specifically recognized protein species in E9 chick ciliary ganglion lysates of 55–65 kDa and 170 kDa, respectively, consistent with the size of human counterparts (Fig. 2A).

Using these characterized antibodies, we analyzed developmental changes in the level of GFR α 1, GFR α 2 and Ret proteins. Two strong bands immunoreactive with GFR α 1 at approximately 55 and 65 kDa were detected in E6 and E9 ciliary ganglion neuron lysates. However, the lower band (\approx 55 kDa) was faint in E12 and E16 ciliary ganglion lysates with the intensity of the upper band remaining strong (Fig. 2B). Western blot analysis with GFR α 1-, GFR α 2- or Ret-transfected fibroblasts indicated that the lower, 55 kDa, band is the specific band for GFR α 1 (data not shown). Noticeable changes in immunoreactivity were also observed with the Ret antibody. An intense immunoreactive band at 170 kDa was observed in ciliary ganglion lysates at E6 and E9, whereas the band of the same molecular mass was very faint at E12 and E16. In contrast to the developmental changes seen with the GFR α 1 and Ret antibodies, the level of GFR α 2 remained relatively constant during a period from E6 to E16 (Fig. 2B). Likewise, CNTFR α and gp130, receptor components for CNTF, remained at a stable level at the embryonic ages tested

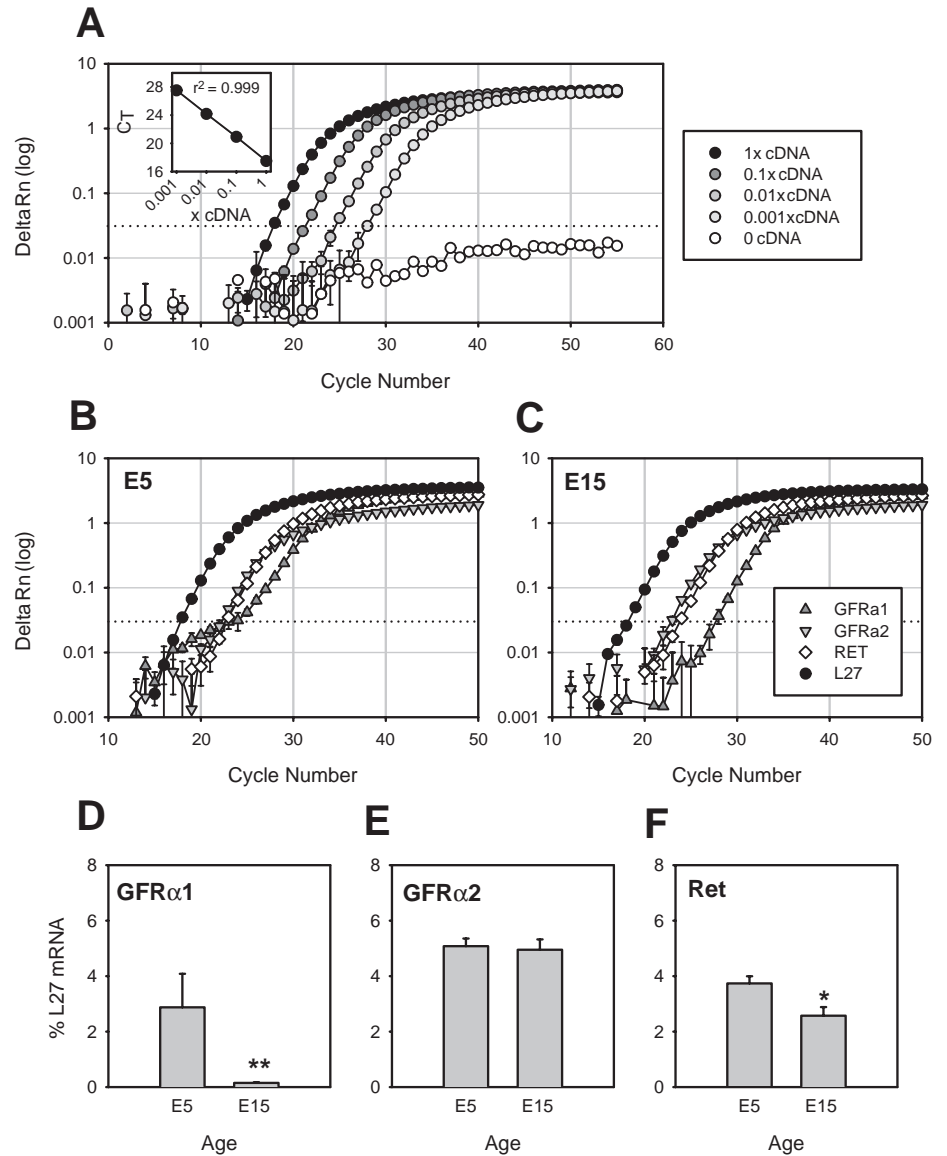
(Fig. 2B). To check if the developmental changes in GFR α 1 and Ret levels are specific to the ciliary ganglion, western blot analysis was also performed with whole brain lysates (Fig. 2B). The intensity of immunoreactive bands for GFR α 1 and Ret in the brain did not change from E9 to E16, confirming that the reduction in receptor expression did not take place globally. The level of GFR α 2 and Ret was very low in the brain. The intensity of the CNTFR α -immunoreactive band in brain lysate became stronger with age from E9 to E16, a trend similar to the one reported for the chick retina (Fuhrmann et al., 1998).

To test whether developmental changes in the expression of GFR α 1 and Ret in ciliary ganglion neurons occur at a transcriptional level, we performed quantitative RT-PCR. We first checked the linearity and the reliability of the detection system that we used (Fig. 3A). Real-time PCR was run with E5 ciliary ganglion neuron cDNA and a primer pair and TaqMan probe for L27, a housekeeping ribosomal gene. At each PCR cycle, the fluorescence intensity (Rn) was measured from quadruplicate samples per probe. At the end of a PCR run (55 cycles), a graph showing the change in Rn as a function of the cycle number was generated. From this graph, the Ct value was calculated as the cycle number at which Rn first exceeds a threshold. The correlation coefficient calculated from 4 different cDNA concentrations was 0.999, demonstrating the linearity of the detection system over the range of 10^4 . Average PCR reaction curves for GFR α 1, GFR α 2, Ret and L27 for E5 and E15 ciliary ganglion neuron cDNA samples are presented in Fig. 3B,C. Comparison of relative mRNA levels between E5 and E15 cDNA samples showed that the mRNA level of GFR α 1 and Ret was significantly higher in E5 ciliary ganglion neurons than in E15 ciliary ganglion neurons (Fig. 3D, F). The GFR α 1 and Ret mRNAs in E5 cDNA sample were 19 times and 1.5 times higher, respectively, than in E15 cDNA samples. In contrast, an equivalent level of GFR α 2 mRNA was detected in both E5 and E15 ciliary ganglion neurons (Fig. 3E).

GDNF and NRTN are synthesized in target tissues of ciliary ganglion neurons during development

To verify *in vivo* biological roles of GDNF and NRTN in ciliary ganglion neuron development, we evaluated the expression of GDNF and NRTN proteins in eyes, the peripheral targets of the ciliary ganglion neurons. Both GDNF and NRTN are expressed in developing eye tissues (Fig. 4A) and, more interestingly, GDNF protein levels changed during development. GDNF was present at E6 and E9, but was absent at E12 and E16. Results from titer experiments indicated that

Fig. 3. Expression of GFR α 1 and Ret mRNAs, but not of GFR α 2 mRNA, is down-regulated in ciliary ganglion neurons during development. (A) Calibration PCR reaction profile with E5 ciliary ganglion neuron cDNA. To check the linearity of the detection system, various dilutions (1, 1:10, 1:100, 1:1000) of E5 ciliary ganglion neuron cDNA as well as water (control) were amplified with a primer pair and a TaqMan probe for L27 housekeeping gene. The correlation coefficient was calculated from the standard curve that displays threshold cycles (C_t) as a function of \log_{10} cDNA concentrations (inset). (B,C) Real-time PCR reactions for GFR α 1, GFR α 2 and Ret mRNA expression in E5 and E15 ciliary ganglion neurons. Real-time PCR for each of the three receptors was run using E5 (B) or E15 (C) ciliary ganglion neuron cDNA samples. For an internal control, the L27 mRNA level was measured with each cDNA sample. Each solid line in the graphs shows the average of quadruplicate PCR reaction profiles. Note that the Y axis in the graphs is in a log scale. A dotted horizontal line in each graph indicates a threshold for that experiment. (D-F) Comparison of the expression of GFR α 1, GFR α 2 and Ret mRNAs between E5 and E15 ciliary ganglion neurons. Based on C_t values obtained from 2 separate experiments, the mRNA level for each probe relative to L27 mRNA level (internal control) was calculated. The GFR α 1 and Ret mRNA levels in E5 ciliary ganglion neurons are approximately 19 times and 1.5 times higher, respectively, than those in E15 ciliary ganglion neurons. **, Significantly different from the E5 group ($P < 0.01$); *, $P < 0.05$.



the detection level of our western blot analysis with the GDNF antibody was approximately 30 pg per lane. Based on a concentration-immunodensity curve, we estimated the GDNF protein in E6 and E9 eye lysates to be higher than 500 pg per lane. Taken together, the GDNF level in the eye at E12 was less than 10% of the GDNF level at E9. The GDNF antibody used in this study did not cross-react with NRTN or ARTN, indicating that these proteins were not detected with the antibody. In contrast to the dramatic temporal changes for GDNF, no noticeable change in the NRTN protein level was observed during a period between E6 and E16. An immunoreactive band with a constant intensity was detected in E6-E16 eye lysates at 25 kDa, a position slightly higher than that for recombinant human NRTN (21 kDa) (Fig. 4A). The difference in the molecular masses for the immunoreactive bands may reflect the fact that recombinant NRTN contains a smaller number of amino acids, therefore migrating faster on SDS-PAGE than chick mature NRTN. In addition, the NRTN antibody did not cross react with GDNF or ARTN, validating the specificity of this antibody.

To identify the cellular source of GDNF and NRTN, we immunohistochemically detected these proteins in chick eye tissues. Transverse sections of E7 chick heads were probed with the same GDNF antibody that was used for western blot analysis. Strong GDNF immunoreactivity was localized to the ciliary muscle near the iris, as well as lateral and medial rectus muscles surrounding the eye (Fig. 4B,C). Examination of higher magnification photographs revealed that GDNF-positive cells in rectus muscle have parallel fibers with distinct transverse bands along the length of fibers, a morphological feature characteristic of striated muscle (Fig. 4C inset). A similar staining pattern with a significantly weaker intensity was seen in tissues stained for NRTN (data not shown).

GDNF and NRTN are secreted as diffusible proteins from eyes during development

To test whether GDNF or NRTN is released as a diffusible protein, we prepared eye-conditioned medium from E5 or E12 chick embryos. In control experiments, we first tested the specificity of a function-blocking antibody to GDNF that we

Fig. 4. GDNF and NRTN are present in the eye during and after target innervation. (A) Western blot analysis for GDNF and NRTN in eye lysates collected from different embryonic ages. The GDNF-immunoreactive band at a similar molecular mass to recombinant human GDNF (rhGDNF) is present in E6 and E9 eye lysates, but is absent in E12 and E16 eye lysates. The anti-GDNF antibody used in this study does not cross react with recombinant human NRTN (rhNRTN) or ARTN (rhARTN). Each recombinant protein (rhGDNF, rhNRTN or rhARTN) was loaded at 1 ng/lane. In contrast to the temporal changes in GDNF, a constant level of NRTN is detected in eye lysates at all developmental stages tested. This anti-NRTN antibody does not cross react with rhGDNF or rhARTN. (B,C) Immunohistochemical localization of GDNF in E7 eye tissues. Strong immunoreactivity was observed in ciliary muscle (B) as well as lateral and medial rectus muscle located along the lateral sides of the eye (C). (Inset in C) A higher magnification photograph of immunoreactive rectus muscle. Scale bars, 200 μ m (B), 500 μ m (C).

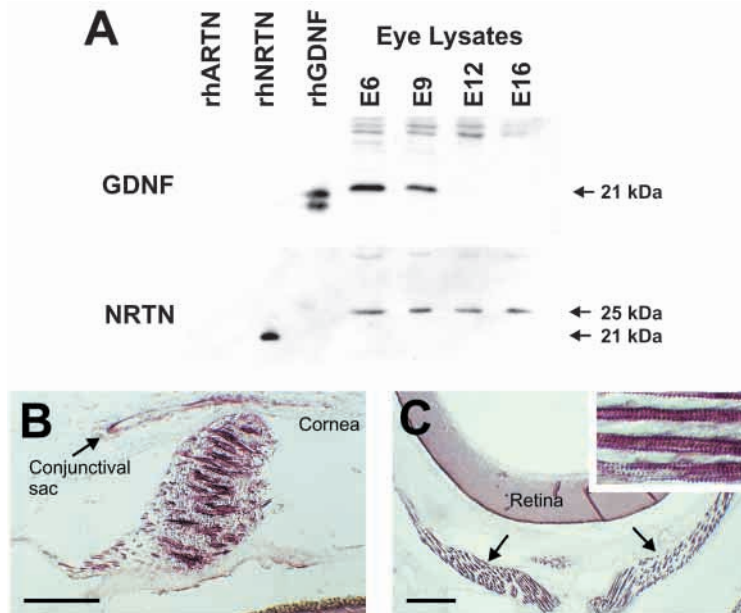
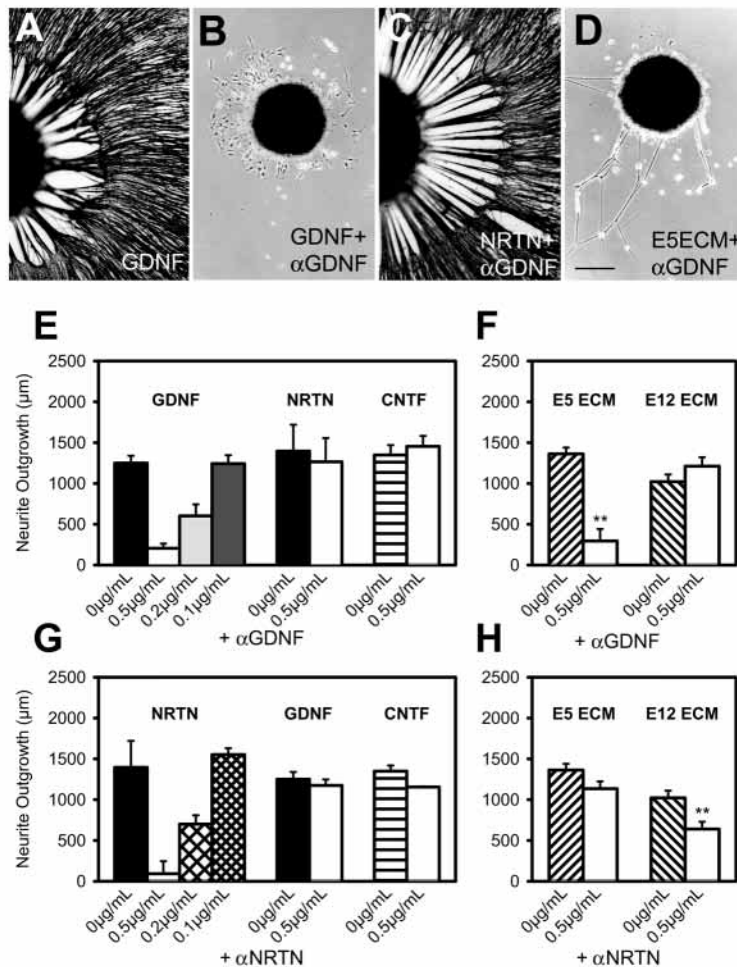


Fig. 5. GDNF and NRTN are secreted from eye tissues during and after target innervation. (A-D) E9 ciliary ganglion explants grown for 2 days in the presence of GDNF (A), GDNF + anti-GDNF antibody (B), NRTN + anti-GDNF antibody (C), or E5 ECM + anti-GDNF antibody (D). Note that anti-GDNF suppresses GDNF-induced neurite outgrowth, but not NRTN-induced neurite outgrowth. Anti-GDNF is able to suppress E5 ECM-induced neurite outgrowth. A and C were immunostained with a TUJ1 antibody and B and D were unstained phase-contrast micrographs. Scale bar, 200 μ m. (E,F) Quantitative analysis of in vitro function-blocking effects by anti-GDNF antibody. (E) Anti-GDNF antibody specifically reduces GDNF-induced neurite outgrowth in a dose-dependent manner. E9 ciliary ganglion explants were grown for 2 days in medium containing 10 ng/ml GDNF and different concentrations of anti-GDNF antibody. The length of neurites (\pm s.e.) extending from treated and untreated ciliary ganglion explants was measured. In another group, explants were grown in medium containing NRTN or CNTF (10 ng/ml, each) with or without anti-GDNF antibody (0.5 μ g/ml). The function-blocking effect by the GDNF antibody is specific to GDNF, and this antibody has no effect on NRTN- or CNTF-induced neurite outgrowth. (F) E5 ECM-induced neurite outgrowth is suppressed by co-incubation with anti-GDNF antibody. In contrast, the GDNF antibody has no effect on E12 ECM-induced neurite outgrowth. (G,H) Quantitative analysis of in vitro function-blocking effects by anti-NRTN antibody. (G) Anti-NRTN antibody reduces NRTN-induced neurite outgrowth in a specific and a dose-dependent manner. The graph shows the average length of neurite (\pm s.e.) extended from ciliary ganglion explants that were cultured for 2 days in medium containing 10 ng/ml NRTN and different concentrations of anti-NRTN antibody. The graph also shows the effects of anti-NRTN antibody (0.5 μ g/ml) on neurite outgrowth induced by GDNF or CNTF (10 ng/ml, each). (H) Anti-NRTN antibody has little effect on E5 ECM-induced neurite outgrowth, but decreases E12 ECM-induced neurite outgrowth. E9 ciliary ganglion explants were grown in medium containing E5 ECM or E12 ECM with or without anti-NRTN antibody. Each bar represents data from 8-14 explants. **, Significantly different from the 0 μ g/ml group ($P < 0.01$).



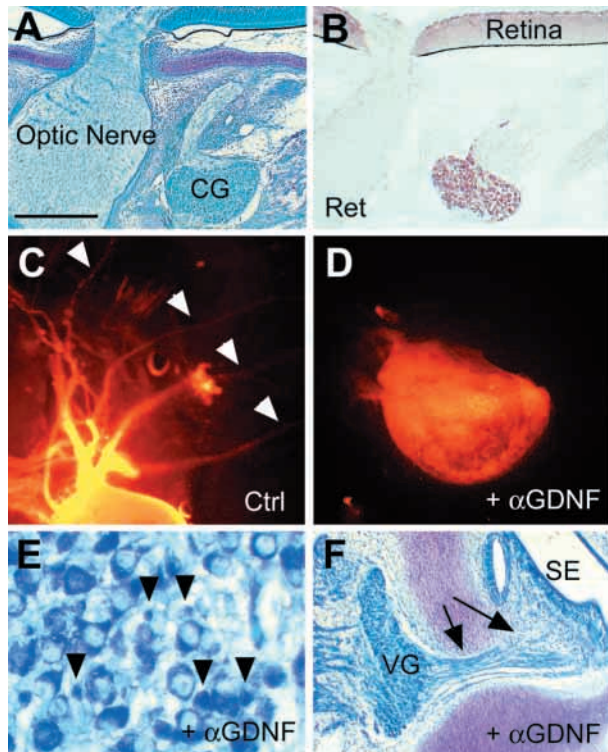


Fig. 6. GDNF is required for target innervation of ciliary ganglion neurons. (A,B) An E7 chick ciliary ganglion stained with Toluidine Blue (A) or anti-Ret antibody (B). The ciliary ganglion is located near the optic nerve and extends its axons in the distal direction. The majority of neurons in the ciliary ganglion at this stage are Ret positive. (C,D) DiI labeling of ciliary ganglion pioneer axons in an E8 chick embryo that was grown for 4 days in vivo in the absence (C) or presence (D) of a function-blocking antibody to GDNF. Growing axons and branches usually seen in control embryos (arrowheads in C) were totally suppressed in embryos that were grown with the exogenously applied GDNF antibody (D). (E,F) Transverse sections of part of the head of chick embryos grown in the presence of the GDNF antibody. (E) Several neurons in the ciliary ganglion exhibit shrinkage of their cell body (arrowheads). (F) Vestibular ganglion axons (arrows) show normal innervation patterns and extend their processes into the vestibular epithelium. Scale bar (shown in A): A,B, 200 μm ; C,D, 400 μm ; E, 40 μm ; F, 250 μm . CG, ciliary ganglion; VG, vestibular ganglion; SE, sensory epithelium.

used. The anti-GDNF antibody suppressed GDNF-induced neurite outgrowth in a dose-dependent manner, reducing approximately 80% of neurite outgrowth at 0.5 $\mu\text{g}/\text{ml}$ (Fig. 5A,B,E). In contrast, this antibody had little effect on NRTN- or CNTF-induced neurite outgrowth (Fig. 5C,E). E5 eye conditioned medium (ECM) promoted neurite outgrowth of E9 ciliary ganglion neurons to a degree comparable to that of 10 ng/ml GDNF (Fig. 5F), whereas E5 otocyst conditioned medium had no effect on the ciliary neuron outgrowth (data not shown). When the anti-GDNF antibody was applied to a culture medium containing E5 ECM, the neurite outgrowth usually seen with E5 ECM was suppressed (Fig. 5D,F). E12 ECM also promoted the outgrowth of E9 ciliary ganglion neurons, but to a lesser extent than E5 ECM (Fig. 5F). The neurite outgrowth promoting effects of E12 ECM, however, were not suppressed by the GDNF antibody.

Table 1. In vivo effects of function-blocking GDNF antibody on ciliary ganglion neuron outgrowth

	<i>n</i>	Outgrowth \pm s.e. (μm)	% relative to control
Control	7	1590 \pm 173	100
+ α GDNF	8	227 \pm 62	14.3

Like the anti-GDNF antibody, a function-blocking antibody to NRTN was found to inhibit the NRTN-induced outgrowth of E9 ciliary ganglion neurons in specific and dose-dependent manners (Fig. 5G). However, the NRTN antibody exerted strikingly different effects on E5 ECM- and E12 ECM-induced neurite outgrowth. This antibody had little effect on the E5 ECM-induced neurite outgrowth, but significantly inhibited E12 ECM-induced neurite outgrowth (Fig. 5H). Although significant, the function blocking effects of the NRTN antibody on E12 ECM was small, reducing neurite outgrowth by only 30%.

GDNF is required for peripheral target innervation of ciliary ganglion neurons

Because the function-blocking antibody to GDNF almost entirely suppressed E5 ECM-induced neurite extending activities in vitro, we wanted to test whether blocking of exogenous GDNF in vivo would have effects on ciliary ganglion neuron innervation patterns. We injected the function-blocking GDNF antibody used in the in vitro experiments into live chick embryos at E3.5, a stage before ciliary ganglion neurons start to extend their axons toward their peripheral targets. The embryos were then allowed to grow in the presence of the antibody for the next 4 days, after which axonal processes of ciliary ganglion neurons were traced with DiI. In control embryos injected with PBS solution, DiI-labeled axon arbors extending toward the frontal direction of the eye were clearly seen (Fig. 6C). In contrast, virtually no processes except for thin short fibers around the ganglion were observed in embryos injected with the anti-GDNF antibody (Fig. 6D). The average length of ciliary ganglion axons in the GDNF antibody-treated group was only 227 μm , compared with 1590 μm in the PBS-treated control group ($t=7.839$, $df=13$, $P<0.001$; Table 1). In some of the ciliary ganglia treated with the GDNF antibody, a small proportion of neurons had shrunken cell bodies, leaving empty spaces in sections (Fig. 6E). In contrast, other cranial ganglia, such as vestibular and trigeminal ganglia, contained normal complements of neuron cell bodies and extended axons into their peripheral and central target tissues (Fig. 6F).

DISCUSSION

GDNF and NRTN are target-derived factors essential for ciliary ganglion axon outgrowth

Although GDNF and NRTN have been shown to exert neurotrophic effects on developing ciliary ganglion neurons in vitro (Buj-Bello et al., 1995; Hashino et al., 1999b), factors released from their target tissues during the period of axon outgrowth and innervation are unknown. We have shown in this study that both GDNF and NRTN are synthesized in and secreted from eye tissues containing all peripheral targets for ciliary ganglion neurons during development. Furthermore, we

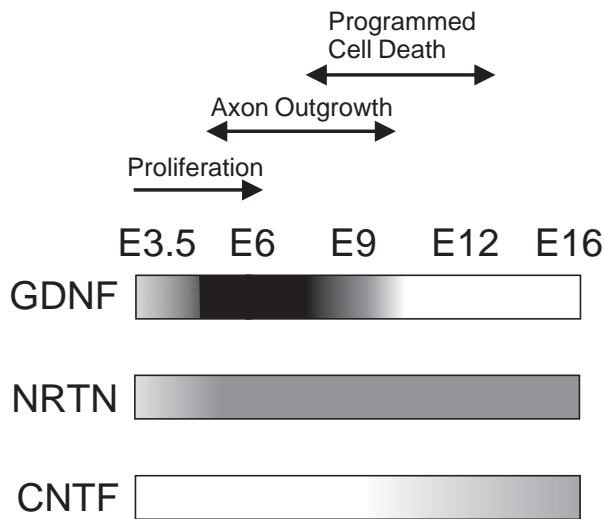


Fig. 7. Schematic diagram showing developmental changes in the synthesis of GDNF, NRTN and CNTF proteins in the chick embryonic eye. The darkest area indicates the highest protein level. The data on GDNF and NRTN are from the present study, whereas the data on CNTF are from Leung et al. (Leung et al., 1992) and Finn and Nishi (Finn and Nishi, 1996).

have found that GDNF and NRTN are synthesized in eye tissues during different developmental periods (Fig. 7). The most striking aspect of this finding is that the two structurally related peptide proteins appear to play distinct roles in ciliary ganglion neuron development. GDNF was present in eye lysates at E6 and E9, but was undetectable at E12 and E16. In contrast, constant levels of NRTN were detected at all developmental stages we tested from E6 to E16 (Fig. 4A). The differential regulation of GDNF and NRTN synthesis in eye tissues was confirmed by *in vitro* function-blocking experiments (Fig. 5). E5 eye conditioned medium promoted the neurite outgrowth of E9 ciliary ganglion neurons. This *in vitro* effect, however, was suppressed almost entirely by a function-blocking antibody to GDNF, but not by a function-blocking antibody to NRTN. These results suggest that GDNF is a major component of proteins resolved in E5-6 eye tissues. Since NRTN protein was detected in E6 eye lysate (Fig. 4), we assume that exogenous GDNF, which was not antagonized by the NRTN antibody, was sufficient to promote the neurite-extending activities of E9 ciliary ganglion neurons. The essential role of GDNF in ciliary ganglion neuron axon development was further verified by *in ovo* application of the GDNF antibody into chick embryos at E3.5, a stage before ciliary ganglion neurons start to extend their axons toward their peripheral targets. The GDNF antibody almost entirely abolished neurite processes, which were observed in PBS-injected age-matched control embryos (Fig. 6C,D).

Immunohistochemical staining localized strong signals for GDNF and, to a lesser extent, NRTN in striate rectus muscle and smooth ciliary muscle of the E7 eye (Fig. 4B,C). This further supports the assumption that GDNF and NRTN are synthesized in the peripheral targets of ciliary ganglion neurons, given that ciliary neurons innervate smooth muscle in the ciliary body (Pilar et al., 1980). In addition, GDNF/NRTN-immunoreactive striate muscle was detected along the trajectory pathways of ciliary ganglion axons. Therefore,

GDNF and NRTN are likely to provide long-distance cues, as well as short-distance cues, during peripheral target innervation of ciliary ganglion neurons. Although the present results clearly indicate that GDNF and NRTN exert axon-extending and survival-promoting effects, the assays used do not address the question of whether GDNF or NRTN provide a directional cue for ciliary ganglion neurons at the beginning of axon outgrowth.

In contrast to the results obtained with E5 ECM, the function-blocking GDNF antibody failed to suppress neurite extending activities of E9 ciliary ganglion neurons that were induced by E12 ECM (Fig. 5F). This is consistent with our western blot analysis, in which GDNF protein was not detected in E12 eye lysate (Fig. 4A), confirming that virtually no GDNF is secreted from eyes at this developmental stage. However, the function-blocking antibody to NRTN was able to suppress E12 ECM-induced neurite outgrowth, albeit to a lesser extent than we expected (Fig. 5H). As GDNF was absent in E12 eye lysate, it is not clear why the NRTN antibody did not totally inhibit E12 ECM-induced neurite outgrowth. Since cDNA for chicken NRTN has not been isolated, we do not know the sequence homology between chicken NRTN and its human counterpart. Thus, one might suggest that the anti-human NRTN antibody used in this study is not able to neutralize chicken NRTN. An alternative possibility is that E12 ECM may contain another neurotrophic factor in addition to NRTN. Support for this hypothesis comes from evidence that detectable levels of CNTF mRNA were expressed in chick eyes at E10 and later stages and that chicken CNTF is a diffusible protein (Leung et al., 1992). Since the CNTF protein level in eyes increased more than 10-fold from E12 to E18 (Finn and Nishi, 1996), we assume that the CNTF protein level at E12 may be very low and that CNTF is not synthesized until most, if not all, of the growing ciliary ganglion axons reached their peripheral targets. Collectively, NRTN may be a major diffusible protein present in eye tissues during the cell death period (E9-E13; Landmesser and Pilar, 1974).

The significance of the differential regulation of GDNF and NRTN in ciliary ganglion neuron development is unclear. However, NT-3 and BDNF, two closely related neurotrophins, have also been shown to be expressed in distinct regions in pathways of trigeminal ganglion axons and to act cooperatively to facilitate axon outgrowth toward their peripheral target (O'Connor and Tessier-Lavigne, 1999). It is thus plausible to hypothesize that two developmental programs independently control the expression and, consequently, synthesis of two neurotrophic factors that signal through common receptors, resulting in similar effects on the developing nervous system. The redundancy in the production of factors that are essential for neuron survival and innervation would guarantee the accomplishment of normal development, and protect the developmental program against potential gene mutation. Alternatively, it is possible that a spatial and temporal distribution of multiple neurotrophic factors may generate subtle concentration gradients throughout the trajectory pathways of developing neurons.

Expression of GDNF receptors is developmentally regulated in ciliary ganglion neurons

A considerable body of evidence has been accumulated to indicate that GFR α 1 and Ret constitute a functional-receptor

complex for GDNF, while GFR α 2 and Ret are the high-affinity coreceptors for NRTN (Jing et al., 1996; Jing et al., 1997; Treanor et al., 1996; Baloh et al., 1997; Creedon et al., 1997; Klein et al., 1997). GFR α 1 and GFR α 2 are also able to bind NRTN and GDNF, respectively, albeit with less efficacy (Baloh et al., 1997; Buj-Bello et al., 1997; Klein et al., 1997). We found in this study that a sharp decline in GDNF synthesis in the eye is accompanied by a down-regulation of its high-affinity coreceptors in ciliary ganglion neurons. The levels of GFR α 1 and Ret proteins were high at E6 and E9, after which they declined through E12 and E16 (Fig. 2). The number of Ret-positive neurons in the ciliary ganglion also declined concomitantly from E9 to E16 (Hashino et al., 1999b). In contrast, the levels of GFR α 2, CNTFR α and gp130 proteins remained constant between E6 and E16 (Fig. 2). Consistent with these data, the level of GFR α 1 and Ret mRNA in E5 ciliary ganglion neurons was significantly higher than that in E15 ciliary ganglion neurons (Fig. 3). A stable expression of GFR α 2 mRNA and protein, however, correlate with a sustained level of NRTN. Together, these results suggest that a decrease in GDNF released from pre-synaptic target cells may trigger a chain of molecular events in post-synaptic neurons, thereby down-regulating the expression of GFR α 1 and Ret. Support for this hypothesis comes from a previous study where an increase in NGF in target tissues was shown to induce up-regulation of NGF receptor mRNA in sympathetic neurons at the transcriptional level (Miller et al., 1991). Likewise, exogenously applied NGF increased NGF receptor mRNA in adult sensory neurons *in vitro* (Lindsay et al., 1990).

What is the molecular basis of such reciprocal ligand-receptor interactions? Several transcription factors have been implicated to act as upstream effectors that directly or indirectly regulate the expression of the neurotrophic factor receptors. Among the candidate transcription factors, retinoic acid regulates the expression of Ret receptors both *in vitro* and *in vivo* (Tahira et al., 1991; Hashino et al., 1999b; Batourina et al., 2001). Interestingly, retinoic acid, alone or together with bone morphogenetic proteins, was shown to induce a substantial increase in GFR α 1 in cultured rat sympathetic neurons, without changing the expression of GFR α 2 and Ret mRNAs (Thang et al., 2000). Phox2a and Phox2b, the paired-homeodomain transcription factors, also control Ret expression (Morin et al., 1997; Pattyn et al., 1999; Stanke et al., 1999). Verification of involvement of these transcription factors in the GDNF-mediated regulation of GFR α 1 and Ret transcription, however, awaits further investigations.

A critical role of GFR α 2 in parasympathetic neuron development has been implicated since a strong parasympathetic phenotype was observed in GFR α 2 null mutant mice (Rossi et al., 1999). One such phenotype is dry eyes due to a lack of parasympathetic innervation to the lacrimal gland. In these mutant mice, a reduction of parasympathetic innervation to the salivary gland was also detected. In addition, GFR α 2 is predominantly expressed in several cranial parasympathetic ganglia, including ciliary, otic, sphenopalatine and submandibular ganglia (Forgie et al., 1999; Enomoto et al., 2000). Our RT-PCR analysis essentially supports these previous studies by showing that GFR α 2 mRNA is expressed in ciliary ganglion neurons at a level higher than GFR α 1 mRNA (Fig. 3). However, our data also reveal that at an early embryonic age the GFR α 1 mRNA level is

unexpectedly high, approximately 60% of the GFR α 2 mRNA level. Thus, in ciliary ganglion neurons, both GFR α 1 and GFR α 2 are likely to participate GDNF- and NRTN-mediated signaling during a period of axon growth, after which GFR α 2 becomes a major receptor in these neurons. The reduction in the survival responsiveness (ED₅₀) to GDNF, but not to NRTN, occurs between E9 and E12 in chick ciliary ganglion neurons (Forge et al., 1999), supporting our assumption.

Conclusions

We have shown that GDNF and NRTN are synthesized in and secreted from ciliary muscle and striated muscle in the eye at a stage that precisely coincides with that at which ciliary ganglion neurons extend their axon processes toward their peripheral targets. We have also demonstrated that the synthesis of GDNF declines markedly at a time when ciliary ganglion axons reach their peripheral targets, while the synthesis of NRTN is maintained throughout the cell death period. Finally, we have presented evidence that the expression of Ret and GFR α 1, with the exception of GFR α 2, decreases concomitantly with the reduction of GDNF, suggesting that target-derived GDNF regulates the expression of its high-affinity coreceptors in innervating neurons. On the basis of these lines of evidence, we conclude that GDNF is a classically defined target-derived neurotrophic factor that plays an essential role in the ciliary axon outgrowth during the period of target innervation. The dramatic down-regulation of GDNF and GFR α 1 in contrast to a stable level of NRTN and GFR α 2 suggests a novel mechanism by which multiple neurotrophic factors and their high-affinity receptors contribute to neural differentiation and survival.

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