

Analysis of function and expression of the chick GPA receptor (GPAR α) suggests multiple roles in neuronal development

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SUMMARY

Growth promoting activity (GPA) is a chick growth factor with low homology to mammalian ciliary neurotrophic factor (CNTF) (47% sequence identity with rat CNTF) but displays similar biological effects on neuronal development. We have isolated a chick cDNA coding for a GPA receptor (GPAR α), a GPI-anchored protein that is 70% identical to hCNTFR α . Functional analysis revealed that GPAR α mediates several biological effects of both GPA and CNTF. Soluble GPAR α supports GPA- and CNTF-dependent survival of human TF-1 cells. In sympathetic neurons, GPAR α mediates effects of both GPA and CNTF on the expression of vasoactive intestinal peptide (VIP) as shown by the inhibition of GPA- and CNTF-mediated VIP induction upon GPAR α antisense RNA expression. These results demonstrate that GPAR α is able to mediate effects

of two neurokines that are only distantly related. GPAR α mRNA expression is largely restricted to the nervous system and was detected in all neurons that have been shown to respond to GPA or CNTF by increased survival or differentiation, i.e. ciliary, sympathetic, sensory dorsal root, motoneurons, retinal ganglion cells and amacrine cells. Interestingly, GPAR α mRNA was additionally found in neuronal populations and at developmental periods not known to be influenced by GPA or CNTF, suggesting novel functions for GPAR α and its ligands during neurogenesis and neuron differentiation.

Key words: growth promoting activity, CNTFR α , ciliary neurotrophic factor, antisense RNA, sympathetic neuron, neurogenesis

INTRODUCTION

Neurotrophic factors strongly influence the development of vertebrate neurons. Initially considered to function only in the control of neuron survival, effects on proliferation and differentiation of neuron precursor cells suggest multiple roles of neurotrophic factors during neuron development. Several groups of factors with neurotrophic actions have been discovered, including the neurotrophins, the insulin-like growth factors, fibroblast growth factors and the family of neurotrophic cytokines (neurokines) (for reviews see Snider, 1994; Rohrer, 1990; Eckenstein, 1994; Hall and Rao, 1992). Growth promoting activity (GPA) is a neurokine isolated from chick embryonic eye and sciatic nerve and characterized initially by its ability to sustain ciliary neuron survival in vitro (Eckenstein et al., 1990; Leung et al., 1992). The timing and localization of its expression in the eye supports the notion that GPA is the target-derived survival factor for chick ciliary neurons (Leung et al., 1992). However, GPA also functions in vitro as a survival factor for sensory and sympathetic neurons (Eckenstein et al., 1990). In addition, GPA affects sympathetic neuron differentiation as demonstrated by the inhibition of prolifera-

tion of E7 sympathetic neurons and the induction of the neuropeptide vasoactive intestinal peptide (VIP) (Heller et al., 1993). GPA also acts, albeit with lower efficiency, on rat sympathetic neurons, inducing cholinergic properties and the expression of several neuropeptides (Heller et al., 1993; Fann and Patterson, 1994). The pattern of biological effects elicited by GPA in avian and mammalian PNS neurons is virtually identical to the effects described previously for mammalian CNTF (Heller et al., 1993; Fann and Patterson, 1994; Ernsberger et al., 1989). The only difference observed was a quantitative difference, i.e. GPA being more active than CNTF on chick neurons (Heller et al., 1993).

Despite the similar biological and biochemical properties of GPA and CNTF (Eckenstein et al., 1990; Leung et al., 1992; Stöckli et al., 1989; Lin et al., 1989), there is evidence to indicate that GPA is not simply the chick homologue of mammalian CNTFs. The deduced amino acid sequence of GPA is only 47% identical to the rat CNTF (Stöckli et al., 1989; Leung et al., 1992), a homology that is much lower than observed between avian and mammalian homologues of neurotrophins (Barde, 1994). Another important difference is that GPA, in contrast to CNTF, is a secreted protein that is released

from transfected cells and from isolated choroid cells in a biologically active form (Leung et al., 1992). GPA and CNTF were also shown to differ with respect to the interaction with high-affinity receptors on chick sympathetic neurons (Heller et al., 1993).

The receptor and signal transduction pathways for CNTF have recently been characterized in detail for mammalian cells and were shown to be related to receptor systems used by a subclass of hematopoietic cytokines ((Squinto et al., 1990; Davis et al., 1991; Davis et al., 1993b) reviewed in Stahl and Yancopoulos, 1994). The action of CNTF is mediated by a heterotrimeric receptor that consists of an extracellular ligand-binding protein (CNTFR α) and two signal-transducing transmembrane proteins, LIFR β and gp130 (Davis et al., 1993b; Stahl et al., 1993). CNTFR α is bound to the cell membrane by a glycosyl-phosphatidyl-inositol (GPI) anchor (Davis et al., 1991). Upon binding of its ligand, the complex of CNTF and CNTFR α is thought to associate sequentially with gp130 and LIFR β to form a functional, signal-transducing CNTF receptor. As the binary complex of gp130 and LIFR β represents the biologically active, high-affinity receptor for leukemia inhibitory factor (LIF), LIF-responsive cells can become CNTF-responsive upon expression of CNTFR α . Interestingly, CNTFR α is able to interact with LIF receptors not only as membrane-bound protein, but also as 'soluble' protein, after release from the GPI-anchor (Davis et al., 1993a). Free, soluble CNTFR α has been detected in cerebrospinal fluid and it is released from denervated skeletal muscle, which may be taken as indication for a physiological role of released CNTFR α (Davis et al., 1993a).

The similar biological effects of GPA and CNTF on avian peripheral neurons strongly suggest that both factors use the same signalling pathways, including gp130 as signal transducing receptor subunit. However, the discrepancies between binding properties and the potency of the elicited biological responses when comparing GPA and CNTF (Heller et al., 1993) raised the question as to the identity of the receptor(s) present in the avian peripheral nervous system and suggested the possible existence of distinct α -receptors that would both signal through gp130. The identification of the receptor mediating the effects of CNTF and GPA was also of interest, since it seemed likely that the analysis of receptor expression would reveal new targets and potential functions, mediated by GPA or related ligands, in addition to the spectrum of biological effects already described for GPA and CNTF (Manthorpe et al., 1982; Nishi and Berg, 1981; Halvorsen and Berg, 1989; Lehwalder et al., 1989; Arakawa et al., 1990; Oppenheim et al., 1991; Heller et al., 1993; Sendtner et al., 1991).

We now report the cloning of a cDNA encoding a GPI-anchored protein that is a functional receptor for GPA (here denoted GPAR α). Both GPA and CNTF are able to mediate biological effects through GPAR α but differ in their relative efficiency as expected from earlier studies (Heller et al., 1993). We provide evidence that GPAR α is an essential component of the endogenous receptor of chick sympathetic neurons, mediating effects of both GPA and CNTF on sympathetic neuron differentiation, as measured by VIP expression. GPAR α is expressed in specific developmental patterns in known target neurons of GPA and CNTF. Interestingly, GPAR α is additionally expressed in several neuronal populations before the period of target-dependent neuronal cell death

and on cells that were previously not known to be targets of GPA or CNTF during development.

MATERIALS AND METHODS

Materials

Recombinant GPA was prepared as described previously (Heller et al., 1993). Recombinant rat CNTF was generously provided by M. Sendtner, Martinsried. Mouse monoclonal antibodies against β -galactosidase and cell proliferation kit (MTT-Assay) were from Promega. Rabbit anti-VIP antiserum was purchased from Incstar. TF-1 cell line (Davis et al., 1993a) and recombinant human CNTFR α were kindly provided by A. Acheson and G. Yancopoulos (Regeneron Inc., Tarrytown).

Cloning of a GPAR α cDNA

An E8 sympathetic neuron cDNA library in the Lambda ZAPII Vektor (Stratagene) was prepared according to the manufacturers specifications. 1×10^6 recombinant phages from the amplified library were screened with a random-primed, ^{32}P -labeled (Feinberg and Vogelstein, 1983) rat cDNA (Huber et al., 1993) spanning the complete coding sequence of the CNTFR α (Davis et al., 1991). Hybridization was carried out overnight at 54°C in $5\times$ SSC, $1\times$ Denhardt's reagent, 20 mM NaH_2PO_4 , 1 mM DTT, 0.1% SDS, 10% Dextran sulfate, 0.1 mg/ml herring sperm DNA. The filters were washed once for 15 minutes in $2\times$ SSC, 0.1% SDS at room temperature and then twice in $1\times$ SSC, 0.1% SDS and in $0.2\times$ SSC, 0.1% SDS at 54°C , respectively. Seven positive clones were purified, in vivo excised (Batzer et al., 1992) and analysed by the dideoxy chain termination method (Sanger et al., 1977) using a T7-Sequencing kit (Pharmacia). Two of the seven clones contained an identical full-length *EcoRI/XhoI*-insert (2.4 kbp), the other five were identical partial clones of the GPAR α sequence. Both strands of the full-length clone were sequenced. Computer analysis of DNA sequences was done with the HUSAR software package (German Cancer Research Center, Heidelberg).

Construction of pCMX-GPAR α , pCMX-antiGPAR α and pcDNA-*lacZ*

The 2.4 kbp *EcoRI/XhoI*-insert of the GPAR α clone was used to replace the *XhoI*-insertion of the human CNTFR α cDNA in the eukaryotic expression vector pCMX-hCNTFR (Davis et al., 1991). We obtained insertions of both orientations, a GPAR α expression plasmid (pCMX-GPAR α) and an antisense RNA expression vector (pCMX-anti-GPAR α). The orientation and identity of the inserts were verified by restriction and complete sequence analysis. For the construction of pcDNA-*lacZ*, a 3.7 kbp *HindIII/BamHI* fragment of the plasmid pSV- β -galactosidase (Promega) containing the coding sequence for β -galactosidase was inserted in the expression vector pcDNAI/Amp (Invitrogen).

Iodination and crosslinking of GPA and CNTF

To obtain high labeling efficiency with ^{125}I , recombinant rat CNTF and GPA with three additional tyrosine residues at the carboxy terminus were used. The modified CNTF and GPA were prepared and purified as described previously (Huber et al., 1993; Heller et al., 1993). The biological activities of the modified forms of GPA and CNTF were verified in survival assays of E8 ciliary neurons and in assays for VIP induction in E7 sympathetic neurons (Ernsberger et al., 1989). The specific activities of modified GPA and CNTF (25 ± 7 pg/ml ($n=5$) and 59 ± 16 pg/ml ($n=4$); mean \pm s.e.m.) were identical to that of the unmodified factors (Heller et al., 1993). Both recombinant proteins were iodinated by a combination of previously published procedures (Rohrer and Barde, 1982; Huber et al., 1993). In brief, lactoperoxidase (Sigma L-2130; 3 $\mu\text{g/ml}$), H_2O_2 (1.8×10^{-9} mol) and 0.4

mCi Na¹²⁵I (Amersham) were added to the modified CNTF or GPA (1 µg) in 40 µl 0.2 M phosphate buffer (pH 7.2) at 0°C. After 30 minutes, a second quantity of H₂O₂ (1.8×10⁻⁹ mol) was added and the reaction continued for additional 2.5 hours. The reaction was terminated by the addition of 40 µl 0.5% NaI in 0.2 M phosphate buffer (pH 7.2) and 4 µl 10% CHAPS. The iodinated factor was separated from free iodine by chromatography over a column containing Sepadex G-50. ¹²⁵I incorporation was determined by trichloroacetic acid precipitation. The specific activity was 200-400 cts/minute/pg protein. The iodinated proteins displayed the same biological activity as the unmodified recombinant factors when analysed for ciliary neuron survival (as shown previously for rCNTF (Huber et al., 1993)).

¹²⁵I-CNTF or ¹²⁵I-GPA were crosslinked to cells growing on tissue-culture plates (3.5 cm diameter for sympathetic neurons; 10 cm diameter for 293 cells) essentially as described by Huber et al. (1993). E10 sympathetic neurons were prepared, purified by preplating and cultured overnight at a density of 3×10⁶ neurons/dish in F14 medium supplemented with 10% horse serum, 5% fetal calf serum and NGF (20 ng/ml) on a laminin-polyornithine culture substrate. 293 cells were transfected (Chen and Okayama, 1987) with pCMX-hCNTFR, pCMX-GPARα or pcDNA-lacZ and cultured for 2-3 days after transfection. Thereafter, the cells were washed once with Krebs-Ringer-Hepes-buffer (KRH; (Rohrer and Barde, 1982)) and then incubated at 4°C for 1 hour with ¹²⁵I-CNTF or ¹²⁵I-GPA (3×10⁻¹⁰ M) in KRH. After this binding period, the buffer was removed and replaced by a solution containing 20 mM of the crosslinker 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDAC) (Calbiochem) in KRH, pH 6.8. The reaction was terminated by adding 100 µl saturated glycine solution per 5 ml incubation volume. The cells were harvested and washed twice by pelleting. The final pellets were lysed in 80 µl of a 0.1% Triton X-100/NaCl/P; buffer (Huber et al., 1993). To remove nuclei, the lysate was centrifuged. The supernatant was combined with double-concentrated PAGE sample buffer, boiled for 5 minutes and loaded onto polyacrylamide gels. After electrophoresis, the gels were dried and exposed to X-ray films.

Production and purification of recombinant 'soluble' GPARα and hCNTFRα

The cDNA-fragments corresponding to amino acid positions 20-342 of the GPARα and 21-342 of the hCNTFRα (Fig. 1) were amplified by PCR and subcloned into the prokaryotic expression vector pASK60-Strep using suggested restriction sites (Schmidt and Skerra, 1993). Using this strategy, the N-terminal signal sequence of the receptor proteins were exchanged to a prokaryotic *OmpA* signal sequence. The resulting vectors were transformed to *E.coli* JM83 and expression of the recombinant receptor proteins was induced with IPTG at 22°C. Since the recombinant proteins are targeted to the periplasmic space, we purified the soluble receptors from the periplasmic fraction of the induced cells using the 'Strep-Tag' strategy according to Schmidt and Skerra (1993). The purity of the preparations was greater than 95% as determined by SDS-polyacrylamide gelelectrophoresis.

Release of GPI-anchored GPARα by enzyme treatment

2 days after transfection (Chen and Okayama, 1987), 293 cells were incubated at 37°C for 1 hour in 100 mM Tris-HCl buffer (pH 8.0) with 6 U/ml phosphatidylinositol-specific phospholipase C (Boehringer Mannheim). The supernatant with the released proteins was concentrated (Centricon, Amicon) and sterilized by filtration (Spin-X, Costar).

GPARα- and CNTFRα-mediated survival of TF-1 cells

The functional properties of soluble GPARα and CNTFRα were analysed using the TF-1 cell survival assay as described previously (Davis et al., 1993a; Panayotatos et al., 1994). TF-1 cells were plated in 96-well tissue culture plates (Falcon) (1×10⁴ cells/well) in the presence of serial dilutions of CNTF or GPA either alone or in com-

bination with soluble GPARα or hCNTFRα. The cells were cultured in RPMI 1640 supplemented with 10% fetal bovine serum and L-glutamine (2 mM). After a period of 48-72 hours the surviving cells were determined using a non-radioactive cell proliferation assay (Mosmann, 1983; MTT-assay; Promega) according to the manufacturers instructions. Triplicate determinations were carried out for each concentration and EC₅₀ values were calculated at half-maximal saturation of the dose-response curves. The addition of GPARα or hCNTFRα results in a shift in the dose-response curve (EC₅₀) that depends on the amount of receptor added and on the ligand present (Panayotatos et al., 1994). The ratio between the GPA-induced shift and the CNTF-induced shift does not, however, depend on the amount of receptor added and has been called relative ligand efficiency. It is also independent of the different ligand potency in the absence of added α-receptors. Soluble GPARα and CNTFRα proteins were produced in *E.coli* and purified by streptavidin affinity chromatography according to Schmidt and Skerra (1993). In some experiments, a different preparation of hCNTFRα (Panayotatos et al., 1994), kindly provided by A. Acheson and G. Yancopoulos) was used.

Transient transfection of primary sympathetic neurons

Cultures of E7 chick sympathetic neurons were carried out as described previously (Ernsberger et al., 1989). After precultivation (4-5 hours), the medium was changed to serum-free conditions and the DNA-precipitate (see below) was added dropwise. The precipitate consisted of 1/40 culture volume DNA in water, 1/40 culture volume 1 M CaCl₂ and 1/20 culture volume 2× BBS (pH 6.94; according to Chen and Okayama, 1987). For the transfection of 1×10⁵ neurons in a 3.5 cm culture dish (Greiner) 20 µg plasmid-DNA was used. After 14-16 hours incubation in decreased CO₂ (3%), the cells were incubated under standard conditions (Ernsberger et al., 1989). 24 hours after transfection, factors were added at concentrations that were just sufficient to induce a maximal proportion of VIP-positive cells after 3 days of treatment (4 days total culture period). At the end of the culture period, the cells were simultaneously stained for β-galactosidase and VIP using a monoclonal mouse anti-βgal antibody (1:1000; Promega) and a polyclonal rabbit anti-VIP antiserum and species-specific FITC- or Texas Red-labeled secondary antibodies as described previously (Ernsberger et al., 1989). Routinely, 2-5% of the neurons are transfected by this procedure as determined with immunostaining for β-galactosidase. The significance of the difference between the results of transfections of different plasmids was determined by Student's *t*-test (pairwise comparison).

Northern blotting

Denatured total RNA was separated on 1% formaldehyde/agarose gels. The RNA was transferred to Hybond-N (Amersham) as described previously (Sambrook et al., 1989). The complete coding sequence of the GPARα was amplified by PCR and a ³²P-labeled probe was made by random priming (Feinberg and Vogelstein, 1983). Hybridization was carried out overnight at 42°C in 5× SSPE, 2× Denhardt's reagent, 0.1% SDS, 50% formamide and 0.1 mg/ml herring sperm DNA. After washing in 0.2× SSC/0.1% SDS at 64°C, the filters were exposed to film for several days. The presence of comparable amounts of RNA per lane was confirmed by reprobing the filters with a chicken β-actin fragment. At least 3 independent experiments were carried out for each tissue analyzed.

In situ hybridization

In situ hybridizations of paraffin sections were carried out as described previously using ³⁵S-labeled RNA probes (Püschel et al., 1992). Only signals detected in several independent experiments and on several sections were considered for the description of transcript expression patterns. The probe used was a partially hydrolyzed antisense-RNA corresponding to base positions 1-1155 of the cloned GPARα cDNA.

[illegible]

Fig. 1. Comparison of the deduced GPAR α amino acid sequence with that of mammalian CNTFR α -proteins. The chick GPAR α amino acid sequence is shown aligned with the sequences for rat and human CNTFR α . The common aminoterminal signal sequence (position 1-21/22) and the hydrophobic carboxyterminal sequence are marked with horizontal lines. Conserved cysteine-residues and the conserved WSXWS motif are printed in bold. Potential N-glycosylation sites are boxed. Identical amino acid residues between chick and human (c:h), and chick and rat (c:r) polypeptides are indicated (*). The putative sites of GPI-anchoring at the carboxyterminal regions of the proteins are printed in bold and underlined. Sequence identities have been optimized by the introduction of gaps (–) and are shown in per cent. The alignments were carried out by using the HUSAR software package (German Cancer Research Center, Heidelberg).

RESULTS

Isolation of a GPA Receptor cDNA

GPA and CNTF differ in their binding properties to receptor(s) on developing sympathetic neurons (Heller et al., 1993). This could be due to a difference in the interactions of the two cytokines with a common receptor or by the co-existence of distinct receptors for GPA and CNTF on sympathetic neurons. As GPA and CNTF are members of a family of neurotrophic cytokines (Bazan, 1991; Patterson and Nawa, 1993; Hall and Rao, 1992) that bind to similar types of receptors (Bazan, 1990; Stahl and Yancopoulos, 1994), we assumed that both the chick GPA receptor and a hypothetical chick CNTF receptor would be related to the mammalian CNTFR α . Thus, a cDNA library, made from embryonic (E8) chick sympathetic neurons, was screened with a rat CNTFR α probe. A plasmid containing a 2.4 kb cDNA insert, including a 159 bp 5' non-translated region and a 1089 bp open reading frame, was isolated. The cloned cDNA encodes a protein of 362 amino acid residues, showing 70% and 68% identity to the amino acid sequence of the human and the rat CNTFR α , respectively (Fig. 1). The encoded protein was named GPA receptor alpha (GPARG α) as GPA is the only ligand known for this protein in the chick. The protein displays the characteristic features of cytokine

receptors, i.e. conserved cysteine clusters of the immunoglobulin-like domain and a cytokine receptor domain including the cytokine receptor consensus sequence WSXWS (Bazan, 1990). Hydropathy analysis revealed a hydrophobic region at its amino terminus, which is typical of secretory signal peptides and a hydrophobic region (see below) after the cytokine receptor-like domain at the carboxyl terminus of the protein. It should be noted, however, that GPAR α contains an additional cysteine residue at position 347 and a deletion of several amino acids in the carboxyterminal region of the protein, as compared to mammalian CNTFR α . The 4 glycosylation sites found in CNTFR α are present in GPAR α at similar positions, but an additional site is found at amino acid 228. This additional glycosylation site may, however, not be functional, as the presence of a neighbouring proline residue is expected to inhibit glycosylation (Gavel and von Heijne, 1990). As described for mammalian CNTFR α , the protein does not contain a transmembrane region but a hydrophobic carboxyterminal sequence indicative for a glycosyl-phosphatidylinositol link of the protein to the cell membrane (Kodukula et al., 1993). Taking into account the rules governing the addition of the GPI anchor to proteins, serine 341 would be the expected carboxy terminal amino acid, resulting in a mature protein of 320 amino acids and a theoretical molecular mass of 36.2×10^3 .

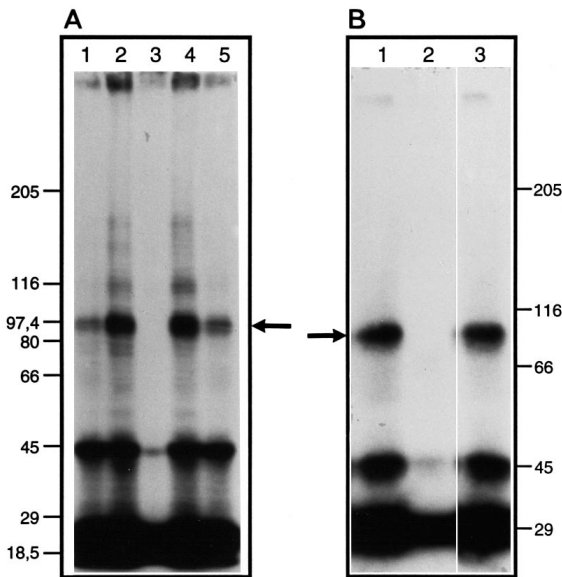


Fig. 2. Crosslinking of bound ^{125}I -GPA (A) and ^{125}I -CNTF (B) to membrane proteins of transfected 293 cells. Cells were transfected with either pCMX-GPAR α (A1, A2, B1) or pCMX-hCNTFR α (A4, A5, B3), and as a control, with the β -Galactosidase expression vector pcDNA-lacZ (A3, B2). Following incubation with radiolabeled GPA (A) and CNTF (B) (both at 3×10^{-10} M), crosslinking and membrane-protein preparation, the equivalents of 5×10^5 cells were separated on SDS-polyacrylamide gradient gels, dried and subjected to autoradiography. ^{125}I -GPA could be crosslinked to avian GPAR α (A2) and human CNTFR α (A4), resulting in a major crosslinked product of about 95×10^3 M_r (arrow). Competition with a 200-fold excess of unlabeled GPA resulted in a decrease of the binding of ^{125}I -GPA on both, GPAR α (A1) and human CNTFR α (A5). No binding of radiolabeled GPA was obtained with 293 cells which have been transfected with pcDNA-lacZ (A3). ^{125}I -CNTF could also be crosslinked to 293 cells which have been transfected with pCMX-GPAR α (B1) or with pCMX-hCNTFR α (B3). In the corresponding control experiment, no crosslinking product was obtained with pcDNA-lacZ transfected cells (B2). Additional bands at about 22×10^3 M_r and 44×10^3 M_r correspond to monomeric and dimerized ligands, respectively. The apparent molecular mass of protein standards is indicated ($\times 10^3$).

Binding of GPA and CNTF to GPAR α

To determine whether the protein encoded by the isolated cDNA can bind GPA or CNTF, we expressed the encoded protein in the human embryonic kidney cell line 293, which does not contain detectable levels of the human CNTFR α (Fig. 2). Following incubation of these cells with either [^{125}I]iodine-labelled GPA (3×10^{-10} M) or [^{125}I]iodine-labelled CNTF (3×10^{-10} M), bound factors were crosslinked to cell surface proteins of transfected cells, using a water-soluble crosslinking agent (EDAC) (Fig. 2). A major crosslinked product of about 95×10^3 M_r was obtained with both GPA (Fig. 2A, lane 2) and CNTF (Fig. 2B, lane 1), indicating that GPAR α binds both GPA and CNTF. Since the molecular mass of GPA and CNTF is about 21.5 and 22.5×10^3 , respectively, the 95×10^3 M_r crosslinking-product corresponds to a 73×10^3 M_r binding protein. The putative GPAR α protein has a calculated molecular mass of 36.2×10^3 , suggesting that GPAR α , like CNTFR α (Davis et al., 1991),

is heavily glycosylated and/or post-translationally modified by other mechanisms.

To compare the binding specificity of the avian GPAR α with mammalian CNTFR α , analogous experiments using human CNTFR α were carried out. The molecular mass of the major crosslinked products obtained with both GPA (Fig. 2A, lane 4) and CNTF (Fig. 2B, lane 3) for human CNTFR α (95×10^3) is virtually identical with the product obtained with GPAR α . Thus also CNTFR α is able to bind both CNTF and GPA.

To control for specificity, binding studies with control transfected cells (Fig. 2A, lane 3; Fig. 2B, lane 2) and incubations in the presence of 200-fold excess of unlabeled GPA (Fig. 2A, lanes 1, 5) were performed. Specific crosslinked products of appropriate molecular mass were detected only in cells transfected with GPAR α or CNTFR α . Binding of ^{125}I -GPA to GPAR α was competed in the presence of an excess of unlabelled GPA. Complete inhibition was not observed and may reflect an inability to reach concentrations required for full competition due to GPA's tendency to aggregate when added at higher concentrations (unpublished observation).

To obtain evidence that GPAR α is indeed the receptor mediating the biological effects of GPA and CNTF on sympathetic neurons, binding of ^{125}I -GPA to E10 sympathetic neurons was investigated in crosslinking studies. After crosslinking of both ^{125}I -GPA and ^{125}I -CNTF to sympathetic neurons, products with the molecular mass of about 95×10^3 were observed (data not shown) which correspond to the crosslinking products obtained with recombinant receptors (Fig. 2). Thus, the binding protein(s) for GPA and CNTF on sympathetic neurons are very similar and comprise a protein with the same molecular mass as GPAR α .

GPAR α is a functional receptor for both GPA and CNTF

To study the function and ligand specificity of GPAR α , a bacterially expressed soluble variant of the GPAR α was produced. It has been shown previously that CNTFR α is able to form a biological active receptor not only when bound to the cell membrane but also as soluble receptor, when liberated from the cell membrane (Davis et al., 1993a). Soluble CNTFR α maintains the relative ligand affinity and specificity of the membrane attached form and acts as the primary determinant of ligand potency (Panayotatos et al., 1994) as it has been demonstrated by using the LIF-dependent erythroleukemic cell line TF-1. TF-1 cells do not express CNTFR α , but a functional LIF receptor, consisting of LIFR β and the signal transduction subunit gp130. They depend on LIF for survival and proliferation and respond, at 100 times higher concentrations, also to CNTF. Soluble CNTFR α , added to TF-1 cells, is able to form functional, high-affinity CNTF receptors consisting of CNTFR α , LIFR β and gp130. As TF-1 cells are able to survive with high concentrations of CNTF in the absence of CNTFR α , the addition of soluble receptor results in a shift of the dose-response curve to lower ligand concentrations (Davis et al., 1993a).

In the absence of added α -receptor both CNTF and GPA induce survival of TF-1 cells at high ligand concentrations; however, GPA is more than 10-fold less efficient than CNTF (half-maximal survival at 420 ± 49 ng/ml (mean \pm S.D.; $n=3$) for GPA as compared to 35 ± 7 ng/ml ($n=3$) for CNTF). The addition of soluble GPAR α strongly potentiates the effects of

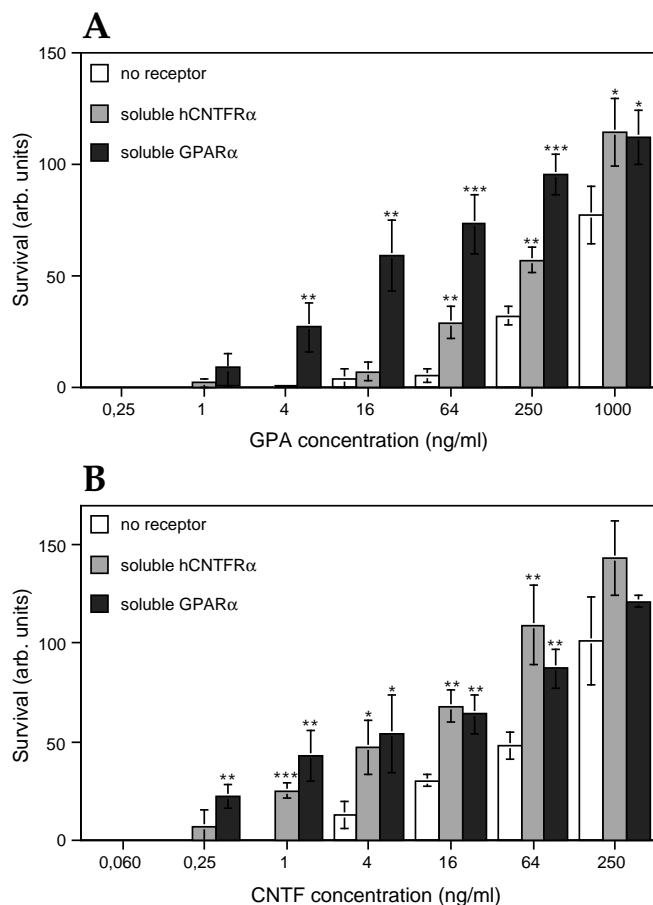


Fig. 3. Soluble variants of GPAR α and human CNTFR α mediate a GPA- and CNTF-dependent increased survival of TF-1 cells. TF-1 cells were incubated with and without soluble GPAR α or hCNTFR α and increasing amounts of either GPA (A) or CNTF (B) were added and the extent of cellular growth was determined by the MTT assay. Data represent the mean \pm S.D. of triplicate determinations in a representative experiment. Stars indicate the significance of the difference between experiments including soluble receptors as compared with the experiments without soluble receptors (***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$; Student's *t*-test (pairwise comparison)). Similar results have been obtained in 2-3 additional experiments.

both GPA and CNTF (Fig. 3). CNTFR α was studied for comparison and also mediated the biological effects of both CNTF and GPA (Fig. 3). The activity of the added receptor can be quantified by determining the extent of the shift of the dose-response curve. As the specific activity of different receptor preparations varied, the properties of GPAR α and CNTFR α were determined by measuring the relative efficiency of signal transduction of a given receptor preparation for the two ligands; this reflects the ratio between the shift in EC₅₀ values elicited by the two ligands (see Methods). GPA was more efficient than CNTF when acting through GPAR α (relative efficiency of GPAR α for GPA compared to CNTF is 3 ± 0.8 (mean \pm S.D.; $n=3$)), but less efficient than CNTF in acting through the CNTFR α (relative efficiency 0.5 ± 0.3 ($n=3$)). The results obtained with human CNTFR α were confirmed with a soluble human CNTFR α preparation, expressed and purified

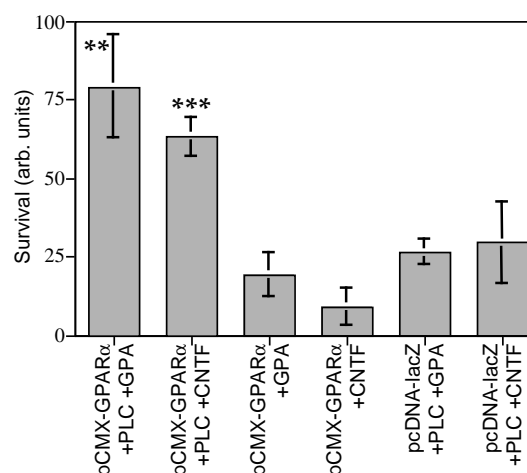


Fig. 4. GPA- and CNTF-dependent survival of TF-1 cells is increased in the presence of PLC-released GPAR α . GPI-anchored proteins were released from 293 cells transfected with either pCMX-GPAR α or pcDNA-lacZ. The transfected cells were incubated with (+PLC) or without phospholipase C and the soluble protein supernatant was used in TF-1 survival assays. In the presence of either GPA (20 ng/ml) or CNTF (20 ng/ml), enhanced survival was only obtained with PI-PLC-digested protein fractions from pCMX-GPAR α transfected 293 cells (pCMX-GPAR α + PLC + GPA or +CNTF). Data represent the mean \pm S.D. of triplicate determinations in a representative experiment. Stars indicate the significance of the experiments including PLC as compared with the experiments without PLC (***, $P < 0.001$; **, $P < 0.01$; Student's *t*-test (pairwise comparison)).

by a different procedure (Panayotatos et al., 1994). Thus, both GPA and CNTF not only bind to but can also functionally interact with GPAR α and CNTFR α and activate the signalling cascade. It should be noted that GPAR α is able to convert cells expressing human LIF receptor into GPA- and CNTF-responding cells, indicating that the protein domains required for the interaction between α -receptors and gp130 have been functionally conserved between GPAR α and hCNTFR α . Despite the complexity of the assay used, involving interactions of receptor subunits of different species, it can be concluded that both α -receptors are able to functionally interact with ligands that display only 47% amino acid identity.

GPAR α is linked to the cell membrane by a GPI anchor

As described for the mammalian CNTFR α proteins (Davis et al., 1991; Ip et al., 1993), GPAR α has no membrane-spanning domain and the C terminus of the protein contains a motif of proteins that are linked to the cell surface via a glycosyl-phosphatidylinositol (GPI) anchor (Kodukula et al., 1993). To investigate if GPAR α is indeed linked to the cell membrane by a GPI-anchor, membranes of GPAR α -transfected 293 cells were digested with a phosphatidylinositol-specific phospholipase C and the solubilized proteins were tested for receptor function. Solubilized proteins from GPAR α -expressing cells, but not from cells transfected with control vectors, are able to mediate GPA- and CNTF-dependent survival of TF-1 cells (Fig. 4). Thus, functional GPAR α can be released from cell membranes by phosphatidylinositol-cleaving enzymes, sug-

gesting that GPAR α is bound to cell membranes through a GPI anchor.

GPAR α antisense RNA expression in sympathetic neurons interferes with the action of both GPA and CNTF

To demonstrate that GPAR α is part of the physiological receptor mediating the biological effects of GPA and CNTF in chick sympathetic neurons, the GPAR α mRNA was reduced in cultured sympathetic neurons by expressing GPAR α antisense RNA in these cells. To identify transfected neurons, an expression plasmid for β -galactosidase (pCDNA-*lacZ*) was co-transfected. GPA or CNTF were added 24 hours after the transfection and the cells were cultured for 3 more days. Transfected cells were identified by immunostaining for β -galactosidase and the proportion of transfected cells that express VIP was determined by double-staining with an anti-VIP antibody.

In cultures of untransfected sympathetic neurons, both factors induced VIP-immunoreactivity (VIP-IR) in about 65% of the cells (Fig. 5). Without factors, no induction of VIP was observed (<1% VIP-IR, see also Ernsberger et al., 1989; Heller et al., 1993). Introduction of the plasmid expressing GPAR α antisense RNA (pCMX-antiGPAR α) into sympathetic neurons significantly interfered with the ability of the neurons to respond to GPA and to CNTF (Fig. 5). VIP-IR was seen in only 24–28% of the transfected sympathetic neurons. The block of biological effects of GPA and CNTF was not complete, as the extent of antisense treatment is probably limited by the turnover of residual GPAR α present on the cell membrane or at the Golgi apparatus at the time of transfection.

To control for the selectivity of the antisense treatment, several control experiments were performed. In antisense-treated cells, addition of soluble GPAR α could partially rescue the ability of the transfected neurons to respond to GPA and CNTF (Fig. 5). Neurons transfected with an expression plasmid for the GPAR α (pCMX-GPAR α) did not alter their responsiveness to GPA and CNTF, suggesting that the number of GPAR α molecules on the cell surface does not limit the ability of E7 sympathetic neurons to respond to GPA or CNTF. We also observed that the population of non-transfected, β -gal-negative cells was not influenced in their ability to respond to GPA or CNTF – the ratio of VIP-induction was about 65% in all cases (data not shown). These experiments exclude unspecific effects of the transfection procedure on the ability of the cells to respond to GPA or CNTF. Furthermore, soluble GPAR α alone did not induce VIP in sympathetic neurons transfected with pCMX-GPAR α (<1% VIP-IR, data not shown).

As an additional VIP-inducing agent, chick heart cell conditioned medium (HCM) was used. Chick HCM contains unidentified factors which are immunologically distinct from GPA and CNTF (Rohrer, unpublished observations). The VIP-inducing effect of chick HCM was not affected by expression of the GPAR α antisense vector. The sustained VIP-inducing effect of HCM excludes the possibility that VIP induction is blocked in a non-specific manner in sympathetic neurons transfected with GPAR α antisense vectors.

The results of the antisense experiments suggest that GPAR α mediates the actions of both CNTF and GPA in chick sympathetic neurons and demonstrate for the first time in

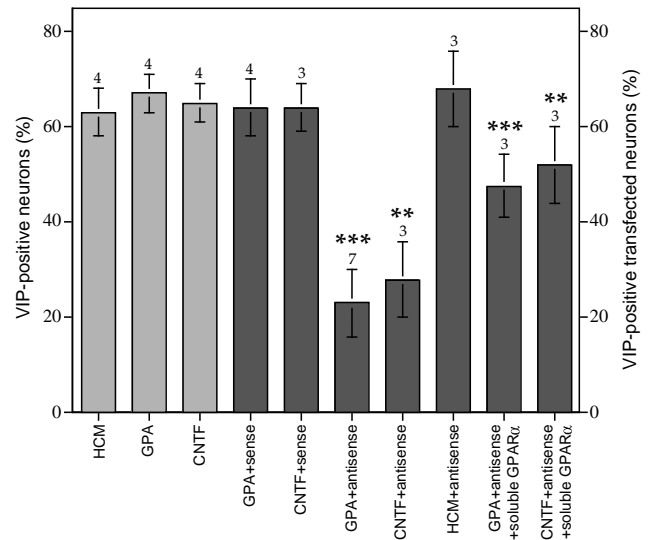


Fig. 5. E7 sympathetic neurons transfected with a GPAR α antisense RNA expression vector exhibit decreased responsiveness to GPA and CNTF. Primary cultures of sympathetic neurons, cotransfected with pCDNA-*lacZ* in combination with either pCMX-GPAR α (sense) or pCMX-antiGPAR α (antisense) or untransfected control cultures were treated with GPA, CNTF or HCM and analysed for VIP expression. Under control conditions GPA (500 pg/ml), CNTF (500 pg/ml) and chicken HCM (present at comparable amounts of biological activity, which has been determined by VIP induction) did induce VIP-immunoreactivity in about 65% of the neurons (HCM, GPA, CNTF). Co-transfected cells were identified by immunostaining for β -galactosidase and the proportion of VIP-immunoreactive, transfected cells was determined. Neurons transfected with a GPAR α antisense RNA expression vector exhibited significantly reduced responsiveness to GPA (GPA+antisense) and CNTF (CNTF+antisense) as compared with either non-transfected cells or with neurons transfected with pCMX-GPAR α (sense), respectively. Data represent the mean \pm S.D. of several independent experiments as indicated by the numbers. Significance values are represented by stars (***, $p < 0.001$; **, $p < 0.01$; pairwise comparisons between sense and antisense experiments, and antisense experiments with and without soluble GPAR α , respectively). Please note that the effect of antisense RNA expression was not observed in the presence of HCM (HCM+antisense), and was partially rescued in the presence of soluble GPAR α (GPA or CNTF +antisense +solubleGPAR α).

primary neurons that the α -receptor is an essential part of the physiological receptor for GPA and CNTF.

Expression of GPAR α mRNA in adult chicken tissues

Expression studies were performed to study the tissue-specific and developmental expression of GPAR α and to define additional potential targets of GPA. The expression of the GPAR α mRNA in selected adult chick tissues was analysed by northern blot hybridization. A single mRNA species of 2.8 kb was detected in all nervous tissues analysed. High levels of GPAR α mRNA were found in cerebellum, brainstem, forebrain, tectum and spinal cord (Fig. 6). Low expression of the GPAR α mRNA was observed in sciatic nerve. Since peripheral nerve contains no neuronal cell bodies, GPAR α mRNA is likely to be

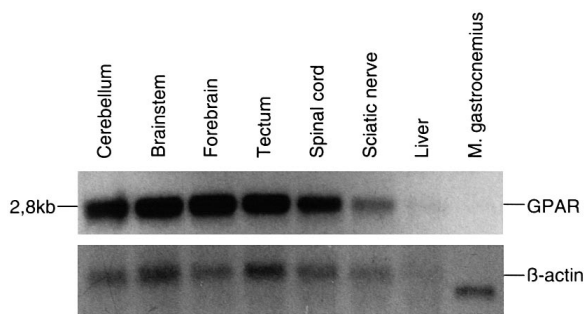


Fig. 6. Northern blot analysis of the expression of GPARα transcripts in adult tissues. Total RNA (10 μg) isolated from the tissues indicated was separated by electrophoresis, transferred to a nylon membrane, and hybridized to a ³²P-labeled GPARα cDNA fragment (complete coding sequence). The blots were rehybridized to a chicken β-actin probe, as a control for RNA loading.

produced by Schwann cells and/or nerve associated fibroblasts. Expression of the GPARα mRNA outside of the nervous system was only detectable at low levels in the liver (Fig. 6). No transcripts were detectable in kidney, lung (data not shown) and, in contrast to the distribution of CNTFRα (Davis et al., 1991; Ip et al., 1993), in skeletal muscle (M. pectoralis and M. gastrocnemius).

Developmental expression of GPARα mRNA

CNTF and GPA have been identified and characterized by their effects on developing neurons in the peripheral and central nervous system. Thus, it was of considerable interest to investigate the expression of GPARα mRNA during development. Northern hybridization demonstrated the presence of significant amounts of GPARα mRNA as early as E3 (Fig. 7). From E5 to E11, GPARα mRNA was found in head and in spinal cord. Whereas GPARα expression in the spinal cord is maintained into adulthood, mRNA levels in the head decreased at E11, which is probably due to the increased contribution of non-nervous tissues. High levels of mRNA were also found in E7/8 sympathetic ganglia, as well as in cultured sympathetic neurons. The highest expression level of GPARα mRNA was observed in E7/8 dorsal root ganglia as judged from the relative intensity of the signals for GPARα and β-actin. Although the expression levels were somewhat lower than for peripheral ganglia, GPARα mRNA was also detected in the eye during early development and expression was maintained in the adult eye (data not shown). As expected for a receptor mediating ciliary neuron survival, GPARα mRNA expression was evident in the ciliary ganglion at all stages analysed between E7 and E14 (Fig. 7). In contrast to adult animals GPARα mRNA could not be detected in embryonic liver.

The localization of GPARα transcripts in the lumbosacral PNS and CNS was examined by in situ hybridization using cross sections of E5 to E12 embryos. High expression of GPARα mRNA was detectable in the ventral horn of the spinal cord between E5 and E8 (Fig. 8A-C). Transcript levels in the motor column peaked at E6 and declined until E12 when hybridization signals were seen throughout the spinal cord (Fig. 8D). High amounts of GPARα mRNA were found in sensory (DRG) and sympathetic ganglia from E5 onwards (Fig. 8A-D). Specific labeling of dorsal root ganglia at low intensity

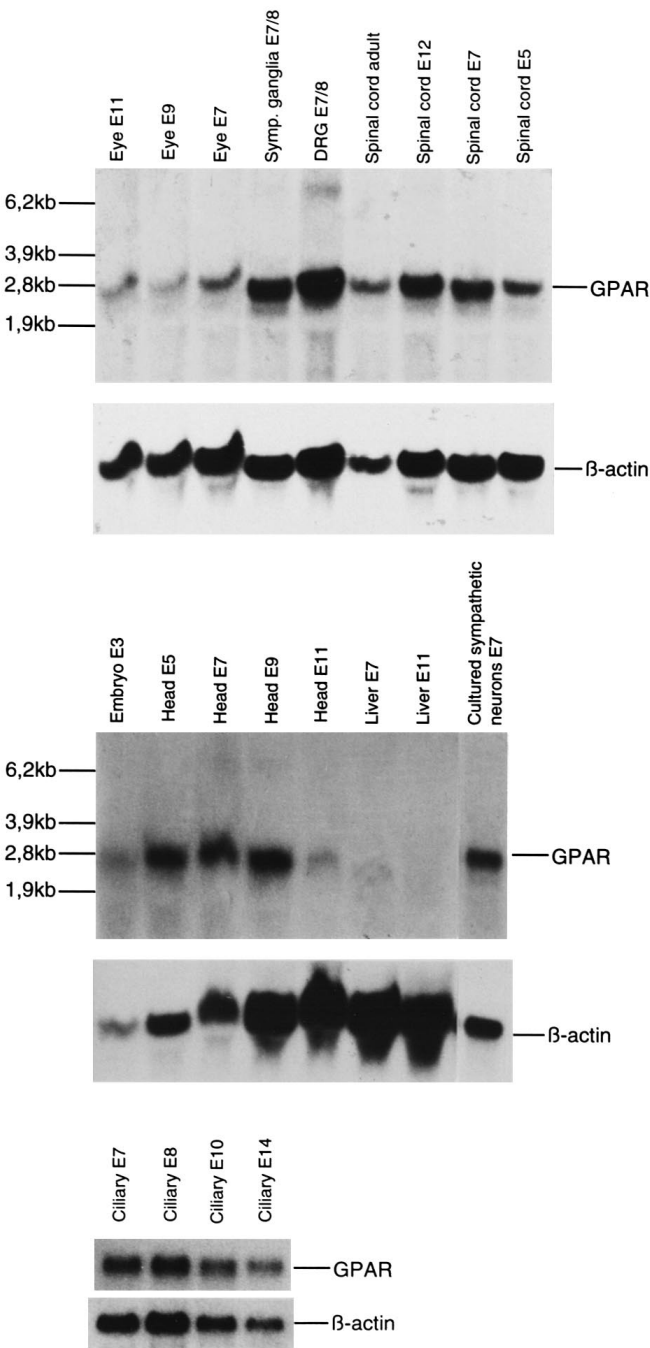


Fig. 7. Developmental expression of GPARα transcripts. Total RNA was isolated from the tissues indicated at different developmental stages and subjected to northern blot analysis (10-30 μg per lane) as described in the legend to Fig. 6. RNA from cultured sympathetic neurons was prepared after 3 days under standard conditions.

was detectable as early as E3.5 (Stage 22, according to Hamburger and Hamilton, 1951; data not shown).

To investigate GPARα expression in the classical target of GPA and CNTF, in situ hybridization studies were carried out using sections of ciliary ganglia from E6, E8, E12 embryos and P16 chicks (Fig. 9). A weak, but distinctive signal was present already at E6 and high levels of expression were obvious from E8 to postnatal stages. The hybridization signals were not

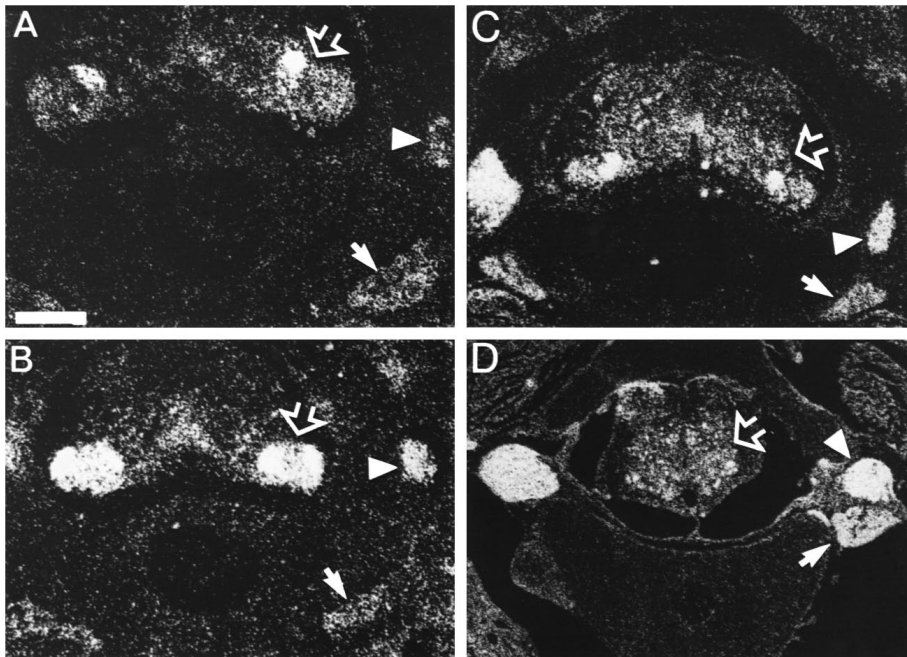


Fig. 8. In situ hybridization analysis of GPAR α mRNA expression in cross sections of chick embryos at the lumbosacral region. Sections from E5 (A), E6 (B), E8 (C) and E12 (D) were hybridized with a ^{35}S -labeled probe for the GPAR α (complete coding sequence). Please note intensive hybridization signals in the ventral horn of the spinal cord (open arrows) and distinct signals in DRG (triangles) and sympathetic ganglia (arrows). The magnification bar corresponds to 150 μm (A), 200 μm (B), 250 μm (C) and 600 μm (D), respectively.

uniformly distributed and areas of low signal intensity seemed to correlate with areas devoid of neuronal cell bodies in sections of E12 and P16 ganglia. However, the resolution of the in situ hybridizations using ^{35}S -labelled probes was not sufficient to assign signals to single cells unequivocally.

Northern blot hybridizations detected GPAR α transcripts also in the developing eye. To investigate this expression in more detail, in situ hybridizations using sections through the central part of the developing retina were carried out (Fig. 10).

Starting at E6, specific signals were observed in the ganglion cell layer and outer marginal zone (Fig. 10A,E). At E8, labeling of photoreceptor cells in the outer nuclear layer (onl), of cells in the inner part of the inner nuclear layer (inl) – where amacrine cells are localized – and in the ganglion cell layer is detectable (Fig. 10 B,F). In the outer part of inl, containing the bipolar, horizontal, and Müller cells, no GPAR α mRNA was detected. The expression of GPAR α mRNA in photoreceptor cells is strongly reduced at E12 (Fig. 10C,G) and is no longer detectable at P16 (Fig. 10D,H). In contrast to the transient expression of GPAR α mRNA in developing photoreceptor cells, GPAR α expression in the amacrine cell layer and in the ganglion cell layer is maintained after hatching (Fig. 10D,H). The spacing of strong hybridization signals in the ganglion cell layer suggests the expression of GPAR α mRNA by subpopulations of ganglion cells or by displaced amacrine cells (Millar et al., 1987; Spira et al., 1987).

DISCUSSION

To understand the signal transduction and the physiological role of neurokinins in the developing chick nervous system, the function and the expression pattern of the GPA receptor were studied. We cloned a chick cDNA that encodes a protein displaying the functional properties of the ligand binding α -component of the GPA receptor complex. The protein sequence is 70% identical to that of the human CNTFR α . An antisense approach provided strong evidence that GPAR α participates in the signal transduction of the endogenous receptor in sympathetic neurons. GPAR α mediates biological effects of the neurokinins GPA and CNTF which are only distantly related (47% amino acid identity). The tissue-specific expression of GPAR α in PNS and CNS both during early development and in adult animals suggests novel functions of this receptor and its ligands in the control of neurogenesis and differentiation in addition to the previously identified roles for the functional

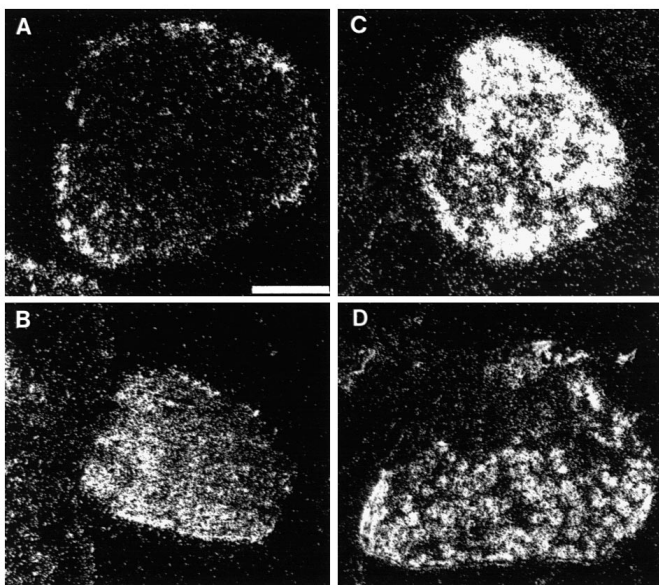


Fig. 9. In situ hybridization analysis of GPAR α mRNA expression in the ciliary ganglion. Sections from E6 (A), E8 (B), E12 (C) and P16 (D) eyes were hybridized with a ^{35}S -labeled probe for the GPAR α (complete coding sequence). Please note strong hybridization signals in ciliary ganglia of E8 to P16 embryos and a weaker, but significant signal at E6. The section shown in B is a peripheral section of the ganglion. The magnification bar corresponds to 29 μm .

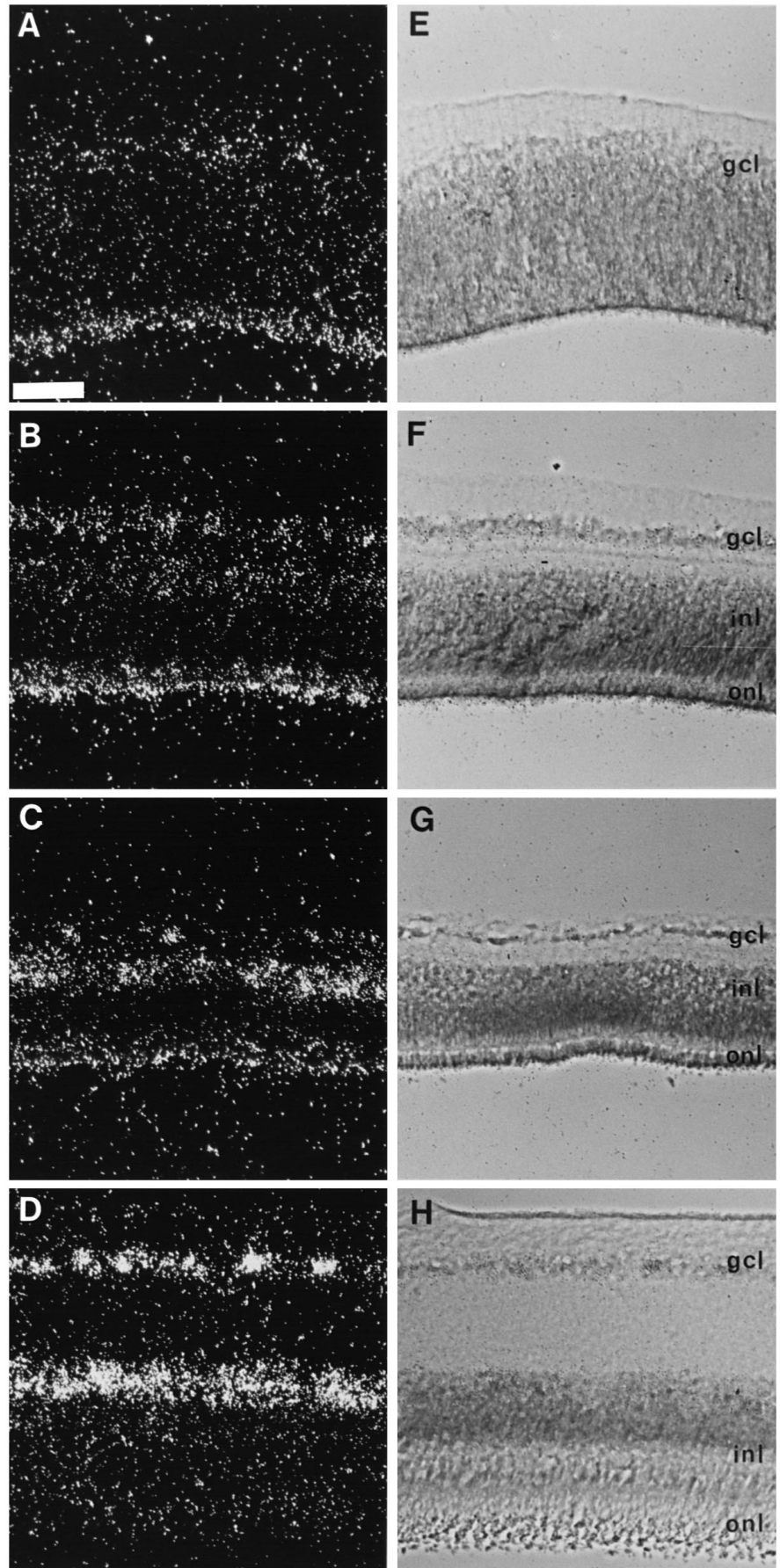
maintenance and the regeneration of neurons in the adult organism.

GPAR α : molecular structure and characterization as receptor for both GPA and CNTF

The cloned cDNA for the chick GPAR α gene codes for a protein that displays a relatively high homology to the amino acid sequence of human and rat CNTFR α (70% and 68%, respectively) (Davis et al., 1991; Ip et al., 1993) and contains the domains characteristic for cytokine receptors (Bazan, 1990). Although GPAR α differs from mammalian CNTFR α by the presence of an additional cysteine and glycosylation site, CNTFR α is the closest relative to GPAR α described so far. The sequence homology to the only other known cytokine α -receptor, human and mouse IL-6R α , is largely restricted to the conserved cytokine receptor domains. In addition, IL-6R α is a transmembrane protein, whereas both GPAR α and CNTFR α are linked to the cell membrane by a GPI-anchor.

Crosslinking studies, using ^{125}I -labeled GPA or CNTF demonstrated that GPAR α binds both ligands. The functional properties of GPAR α , as part of the signal transducing receptor complex, were investigated using a 'soluble', not membrane-bound form of the protein. 'Soluble' forms of IL-6R α and CNTFR α are the only receptors known that are able to mediate the biological action of their ligand rather than to act as competitors (Taga et al., 1989; Davis et al., 1993a; Ip et al., 1993). This is due to the sequential assembly of ligand-bound α -receptors with either a homodimer of the signal-transducing transmembrane glycoprotein

Fig. 10. In situ hybridization analysis of GPAR α mRNA expression in the developing retina. Sections from E6 (A,E), E8 (B,F), E12 (C,G) and P16 (D,H) eyes were hybridized with a ^{35}S -labeled probe for the GPAR α and counterstained with thionin. (A-D) Dark field, (E-H) phase contrast. Please note faint staining in the ganglion cell layer (gcl) and the outer margin of the retina at E6 (A,E), strong labeling of gcl and amacrine cell layer (inner part of inner nuclear layer, inl) at E8, E12 and P16. The outer nuclear layer (onl) displays a hybridization signal at E8 and E12, but not at P16. The pigment epithelium has been separated from the neural retina and is not present in the sections shown. The sections are from the central part of the retina, near the entry of the optic nerve. The magnification bar corresponds to 22 μm .



gp130, or a gp130/LIFR β heterodimer to form functional high-affinity receptors for IL-6 or CNTF, respectively (Davis et al., 1993b). Using a LIF-dependent cell line (TF-1 cells) expressing gp130 and LIFR β but not CNTFR α , as an assay system (Davis et al., 1993a; Panayotatos et al., 1994), we could demonstrate that the soluble GPAR α is a functional receptor for both GPA and CNTF. We could also show that hCNTFR α mediates a response to GPA. The relative efficiencies of the ligands at the receptors expressed in this model system are in agreement with their relative potencies on chick or rat primary culture neurons, GPA being more potent than CNTF in inducing VIP on chick sympathetic neurons, but less active on rat sympathetic neurons (Heller et al., 1993).

These observations indicate that both receptors are able to interact functionally with ligands that display only very limited homology in protein sequence. This is in accordance with the hypothesis proposed by Bazan (1991) that the members of the family of neuropoietic cytokines (neurokines) are characterized by conserved 3D structure but low conservation of primary structure. It should also be pointed out that the TF-1 survival assay requires the interaction of chick GPAR α with human gp130 and LIFR β to form a signal transducing receptor complex, demonstrating that this interaction has also been conserved.

GPAR α is essential for mediating the effects of GPA and CNTF in chick sympathetic neurons

To show that GPAR α is an essential component of the receptor in chick neurons, we inhibited the GPA- and CNTF-mediated induction of VIP in cultured sympathetic neurons by the expression of GPAR α antisense RNA. The selectivity of the antisense treatment is clearly demonstrated by three different control experiments. (i) Expression of GPAR α sense mRNA had no effect. (ii) GPA and CNTF responsiveness in antisense-treated cells could be rescued through the addition of soluble GPAR α . This shows that the functional block in antisense-treated cells is due to a reduction of the receptor levels. The incomplete rescue may be explained by the reduction of efficient concentration of GPA and CNTF by binding to soluble GPAR α not interacting with the receptor complex. (iii) VIP could still be induced in GPAR α antisense-treated cells by HCM, indicating that the signalling cascade eliciting VIP expression is still intact in these cells. This result also indicates that HCM contains VIP-inducing activities that are not mediated by the GPAR α . Since LIF is a major component of rat HCM (Yamamori et al., 1989), the VIP-inducing effect of chick HCM may be due to the presence of the chicken equivalent for LIF.

The inhibition of both GPA and CNTF response supports the conclusion that GPAR α mediates the action of both ligands. However, it is possible that two distinct, but closely related, receptor mRNAs for CNTF and GPA are eliminated by the antisense treatment. We consider this as unlikely, since we obtained no evidence for a closely related gene in a low-stringency northern hybridization analysis on all tissues studied (Heller and Rohrer, unpublished data). Our conclusion is supported by the finding that in CNTFR α (–/–) mice the CNTF signaltransduction pathway cannot be activated by CNTF (DeChiara et al., 1995), suggesting that, for the mammalian system, there is only one receptor mediating CNTF effects. The previously observed lower potency of GPA to compete with CNTF for binding to CNTF-high-affinity-binding sites on sympathetic neurons (Heller et al., 1993) may

be explained by assuming that GPA is more efficient in signal transduction through the heterotrimeric GPA receptor than CNTF, although it binds less well than CNTF to the receptor. There are indeed precedences for a lack of correlation between receptor binding and signal transduction efficiency (Kramer et al., 1994).

GPAR α expression during early PNS and CNS development suggests a role during neurogenesis

GPAR α is mainly expressed in embryonic and adult nervous tissue, in both neurons and non-neuronal cells (sciatic nerve). GPAR α expression precedes the period of neuronal death and is maintained at later developmental stages.

In sensory DRG and sympathetic ganglia, we observed strong expression already at very early stages of neuronal development when neuron precursor cells in these ganglia still divide (e.g. at E5 and E6) (see also Ip et al., 1993). CNTF and GPA inhibit the proliferation of immature E7 chick sympathetic neurons (Ernsberger et al., 1989; Heller et al., 1993) and CNTF collaborates with FGF and NGF to drive terminal differentiation of a sympathetic neuron progenitor cell line (Ip et al., 1994). As CNTFR α and GPAR α are present during neurogenesis in rat and chick sensory and sympathetic ganglia, GPAR α -mediated signals may be involved in the control of neuron proliferation and/or may contribute to the initial differentiation of neuronal precursor cells.

After neurogenesis, during the period of neuronal cell death, GPAR α mRNA was present in ciliary ganglion neurons, spinal cord motoneurons, retinal ganglion cells, sensory DRG neurons and sympathetic neurons, supporting the notion that the survival effects of GPA and CNTF observed on these cells (Arakawa et al., 1990; Oppenheim et al., 1991; Lehwald et al., 1989; Manthorpe et al., 1982; Sendtner et al., 1991) are mediated by GPAR α .

The maintenance of GPAR α mRNA expression after the period of neuronal cell death, as observed for instance in ciliary neurons, retinal ganglion cells, amacrine cells and motoneurons suggests that GPAR α mediates functions in neuronal differentiation at later stages of development. There are several observations supporting this conclusion. GPA and CNTF induce VIP in cultured sympathetic neurons (Ernsberger et al., 1989; Heller et al., 1993) and CNTF stimulates cholinergic differentiation (choline acetyltransferase, ChAT) of E8 retinal cells in vitro (Hofmann, 1988). The expression of GPAR α in sympathetic ganglia and in the amacrine cell layer, where the only ChAT-expressing cells in chick retina (Millar et al., 1987; Spira et al., 1987) are located, suggests that GPAR α may be involved in the induction of VIP and ChAT in these neuronal populations.

The transient presence of GPAR α mRNA in photoreceptors of the developing retina is an unexpected finding since only a protective action of CNTF against damaging effects of light in adult rats (LaVail et al., 1992) has been described for CNTF with no effects on photoreceptor development. As GPAR α mRNA expression is highest at a stage when photoreceptor generation has ended (Prada et al., 1991), it seems possible that GPAR α is involved in photoreceptor survival or in some aspect of photoreceptor differentiation. Interestingly, recent studies by two independent groups demonstrate in vitro effects of CNTF on photoreceptor differentiation (H.-D. Hofmann and C. Cepko, personal communication).

It should also be pointed out that GPAR α mRNA is maintained in mature, fully differentiated neurons, as expected from the known effects of CNTF on rat motoneuron (Sendtner et al., 1990; Sendtner et al., 1992) and retinal ganglion cell regeneration (Mey and Thanos, 1993). The expression of GPAR α mRNA in the adult chick closely resembles the mature expression pattern of mammalian CNTFR α (Davis et al., 1993a; Ip et al., 1993) but differs in the absence of GPAR α mRNA in skeletal muscle.

How many ligands act through GPAR α ?

Compared to the data available on the expression of GPAR α and CNTFR α in the developing and mature avian and mammalian nervous system, our knowledge about the localization and developmental expression of GPA and CNTF is restricted to few tissues that have been investigated in detail (Sendtner et al., 1994; Leung et al., 1992; Finn and Nishi, 1993, 1994). Although very low levels of CNTF were detectable in E14 rat embryos (Ip et al., 1993; but see Stöckli et al., 1991), the major increase in CNTF expression occurs postnatally (Stöckli et al., 1991). As the highest levels of CNTF are present in peripheral nerves, it has been proposed that the major function of CNTF may be to stimulate neuron survival and regeneration upon nerve lesions (Sendtner et al., 1994). High level expression of GPA in adult sciatic nerve (Eckenstein et al., 1990; Leung et al., 1992) suggest a similar function in the chick. In addition, however, there is evidence for expression of GPA in the target tissues of ciliary ganglion neurons (Leung et al., 1992; Finn and Nishi, 1993, 1994). The observed correlation between GPA expression and the onset of naturally occurring cell death supports the notion of a physiological role for GPA as target-derived ciliary neurotrophic factor. The GPA levels in target tissues of other GPA-responsive neurons, including cholinergic sympathetic neurons, are unknown. The previously described absence of GPA mRNA in the chick neural retina (Leung et al., 1992) indicates that the levels are either very low, or that other ligands for GPAR α are present in this tissue. There is indeed circumstantial evidence for the existence of at least two CNTF-like factors from the analysis of transgenic mice. Whereas the elimination of the CNTF gene in transgenic animals has no obvious effects on prenatal development of the nervous system (Masu et al., 1993; DeChiara et al., 1995), strong neuronal deficits were observed in newborn CNTFR α knockout animals (DeChiara et al., 1995). This result indicates that an additional factor, acting through the CNTFR α , is present and essential for nervous system development in the mammalian embryo. Our results, showing that both GPAR α and CNTFR α are able to mediate effects of different only distantly related members of the neurotrophin family, are in agreement with the conclusions from the CNTFR α knockout experiments. It is presently unclear, whether the additional factor acting at the CNTFR α during mouse nervous system development represents the mammalian homologue of GPA or represents an additional new factor, which also exists in chick.

Although the identity of the ligands acting at GPAR α in different neuronal populations and during specific developmental periods still has to be clarified, the present analysis has provided a framework to investigate the function and expression of GPAR α ligands. In addition, the possibility of interfering with GPAR α functions by antisense techniques,

together with the availability of avian retroviral expression vectors, provides the potential to elucidate the role of GPAR α -mediated signals at specific time points of avian nervous system development.

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Note added in proof

The nucleotide sequence data reported in this paper will appear in the EMBL, GenBank and DDBJ Nucleotide Sequence Databases under the accession number Z48168, GGGPARA.