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Effects of GGF/neuregulins on neuronal survival and neurite outgrowth correlate with erbB2/neu expression in developing rat retina

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SUMMARY

We have examined the expression of neuregulin and its putative receptors, erbB2/neu, erbB3 and erbB4/tyro2 during retinal development, and tested several potential functions of this class of molecules in dissociated rat retinal cell cultures. At least one form of neuregulin is expressed in the retina, from the earliest stages of retinal development examined; in addition, all three of the known receptors are expressed by retinal neurons in a developmentally regulated manner. When added to cultures of embryonic or neonatal rat retinal cells, neuregulin (rhGGF2) promotes survival and neurite extension from retinal neurons in a dose-dependent manner. These results indicate that in addition to their well described effects on glia, the neuregulins also have direct effects on central nervous system neurons.

Key words: retinal ganglion cells, ARIA, heregulin, neu, erbB

INTRODUCTION

Neuregulins are a class of cell surface and secreted proteins with diverse functions in a variety of different tissues (see Peles and Yarden, 1993; Mudge, 1993 for review). These factors have been isolated from a number of different cell types, and at least ten different proteins are known to be generated from a single gene (Marchionni et al., 1993). The widespread distribution of these factors and their putative receptors in the developing and mature nervous system is consistent with evidence for a variety of actions for neuregulins (Chen et al., 1994; Corfas et al., 1995; Meyer and Birchmeier, 1994; Jo et al., 1995).

Neuregulins have been shown to have effects on several different types of glial cells. One of the neuregulins, glial growth factor (GGF), was initially identified for its mitogenic actions on Schwann cells (Brockes, 1987). Neuregulins have also been shown to promote astrocyte survival and differentiation in cerebral cortical dissociated cell culture (Pinkas-Kramarski et al., 1994), and to enhance the development of oligodendrocytes from bipotential (O2A) glial progenitor cells (Vartanian et al., 1994). At an earlier stage of neural development, neuregulins promote glial differentiation and inhibit neuronal differentiation in cultured neural crest stem cells (Shah et al., 1994).

While most of the reported effects of the neuregulins are on non-neuronal cells, there is also evidence that they may have direct actions on neurons. First, at least one of the neuregulin receptors, erbB4/tyro2, is expressed by neurons in many different regions of the brain (Lai and Lemke, 1991). Second, the early time course of expression of at least some forms of neuregulin suggest that the molecules may also have effects on neuronal differentiation (Corfas et al., 1995). For example, neuregulin is expressed by ganglion cells in the developing retina prior to the generation of retinal Muller glia (Meyer and Birchmeier, 1994). Third, nARIA, a neural specific form of neuregulin, can stimulate neurite outgrowth and differentiation in PC12 cells (Corfas and Fischbach, personal communication) and regulate the expression of specific subtypes of neuronal nicotinic receptor subunit genes in the CNS (L. Role, personal communication).

To directly test for actions of neuregulins on neurons in the developing central nervous system, we have studied a particularly well characterized region of the CNS, the neural retina. In this study, we have examined the cellular localization of putative neuregulin receptors in the developing retina, and tested whether neuregulins promote neuronal differentiation and/or survival in rat retinal neuron cell cultures. We have found that at least one form of neuregulin is expressed in the retina, and that all three of the reported receptors for these molecules are also synthesized in the retina in a developmentally regulated manner. We have also found that neuregulin can promote neuronal survival and neurite outgrowth in dissociated cell cultures of embryonic and newborn retina, indicating that neuregulins may have important roles in neuronal, as well as glial, development.

MATERIALS AND METHODS

Immunohistochemistry

Long Evan's rats (obtained from Simonsen labs) were used for these experiments. All procedures were carried out in accordance with approved animal protocols at the University of Washington.

Following CO_2 overanaesthesia, the eyes from rats at embryonic days 15 and 18, and postnatal days 2, 5, and 15 and adults were dissected and fixed in 4% paraformaldehyde for 2 hours to overnight. Following fixation, the eyes were rinsed in saline, cryoprotected with sucrose and sectioned on a cryostat at 10 μm. The sections were incubated with antibodies for neuregulins and their receptors, as well as for several of the neuronal-specific antibodies used to label the cells in the dissociated cell cultures (see below).

The antibodies we used in the experiments were either generated at Cambridge NeuroScience or obtained commercially. Affinity purified antibodies raised against different regions of neuregulin were used to localize the protein in the developing rat retina. Antibody 2861 is a polyclonal rabbit antiserum raised against a peptide in the N-terminal region of rhGGF2. Antibody 11366 is a polyclonal rabbit antiserum raised to a peptide in the region of the EGF domain that is conserved in all the neuregulins. Antibody CN16 is a rabbit polyclonal raised by immunizing with the entire rhGGF2 protein.

In addition, several different antibodies raised against the known neuregulin receptors were also used in these studies. Three antibodies raised against erbB2/neu were obtained commercially and used at a 1:500 dilution (AB4, Oncogene Sciences; 9G6, Santa Cruz; K15, Santa Cruz). Rabbit polyclonal antisera specific for erbB3 and erbB4/tyro2 were obtained from Santa Cruz, and used at 1:500 and 1:100 dilutions, respectively. The antibody against erbB3 was a rabbit polyclonal IgG raised against a peptide containing amino acids 1307 to 1323 of the human erbB3 protein. The antibody has been previously shown to cross react with rat erbB3 and does not cross-react with other known members of the EGF-receptor family. Three different erbB4 antibodies were used in this study. Commerically obtained erbB4 antibody was a rabbit polyclonal IgG raised against a peptide containing amino acids 1291 to 1308 of the human erbB4 protein. This antibody has also been shown to cross-react with rat erbB4, but not other members of the EGF-receptor family. Two additional erbB4 mouse monoclonal antibodies were a gift from Drs Clay Siegall, Perry Fell and Bruce Cohen at the Bristol-Myer-Squibb research Institute (Seattle, WA) and were used to confirm the results obtained from the Santa Cruz antibody (Siegall et al., 1995). Following a 2 hour to overnight incubation with the primary antiserum, binding was visualized with biotin-avidin linker secondary antibodies (Vector Labs) and used according to the manufacturer's directions. When available, preimmune serum was used as a control; for those antibodies where preimmune serum was not available, omission of the primary antiserum from the protocol served as a control. We carried out preabsorbtion controls for erbB3 and CN16 by incubating the antisera with the immunizing peptide erbB3 and rhGGF2 for CN16.

Western blotting

Membrane fractions of retina and brain were prepared as follows (modified from the method of Temple and Davis, 1994). Adult rat brain and retinas were dissected in HBSS+ and rinsed in calcium/magnesium-free phosphate-buffered saline (CMF PBS) (Delbecco's Phosphate Buffered Saline, Gibco BRL) and homogenized in 50 mM Tris, 10 mM $MgSO_4$, and 0.5 mM EDTA, pH 7.8. Cells were then centrifuged at 1500 rpm for 5 minutes to pellet whole cells and debris. Supernatant was collected and subjected to 30,000 ***g*** for 1 hour. The pellet was rinsed in homogenization buffer and membranes were collected by centrifugation. The pellet was resuspended in a small volume of CMF PBS and the protein concentration was determined by BCA protein assay (Biorad). Retinal homogenate was prepared by cell lysis (modified from the method of Sambrook et al., 1989). Retinas were dissected in HBSS+ put into 1.5 ml centrifuge tubes containing 100 μl lysis buffer (50 mM Tris (pH 8.0), 150 mM NaCl, 0.02% sodium azide, 100 mg/ml PMSF, 1 mg/ml aprotinin, 1% NP-40) and placed on ice for 20 minutes. The extract was cleared by centrifugation at 15000 rpm for 10 minutes and diluted in 2× sample buffer.

The retina and brain membrane fractions (approx. 40 mg/ml protein concentration) were analyzed on 7.5% acrylamide/bis SDS-PAGE gel and transferred to nitrocellose by standard methods. Proteins were detected by a modified ECL method (Amersham). Briefly, blots were blocked in 5% milk/PBSTween or 0.1% egg albumin/PBSTween for 1 hour. Primary antibodies were diluted 1:1000 in 5% milk/PBSTween and blots were incubated for 2 hours. Blots were then washed with PBSTween three times in 45 minutes. Secondary antibody (HRP goat anti-rabbit IgG (H+L), Zymed) was diluted 1:1500 in 5% milk/PBSTween and blots were incubated for 45 minutes. Blots were washed with PBSTween three times in 15 minutes and then exposed to ECL reagents for 1 minute. Blots were then used to expose ECL X-ray film.

For the phosphotyrosine blots, newborn rat retinas were dissociated as described for the cell cultures and 2.5×10^6 cells plated onto uncoated 25 cm^2 flasks in serum-free medium. After 24 hours, the medium was aspirated and either replaced with fresh medium or fresh medium containing 25 ng/ml rhGGF2. Incubation was continued for a further 2.5 minutes and then the medium removed and the cells harvested into 500 μl of NP40 lysis buffer (20 mM Tris, pH 7.5, 150 mM NaCl, 1% NP40, 0.5% sodium deoxycholate, 1 mM PMSF, 2% aprotinin and 1 mM orthovanadate). The cell lysate was boiled for 10 minutes, spun in a microfuge for 5 minutes at 4°C and stored at –70°C. Equal amounts of extract from treated and untreated cells were seperated by SDS-PAGE and transferred to nitrocellulose. Proteins containing phosphotyrosine were detected by incubating the blot with the RC20 antiphosphotyrosine antibody (Transduction Labs) and subsequent development using ECL.

In situ hybridization

Sense and antisense digoxigenin (Boehringer-Mannheim Biochemicals) RNA probes were synthesized, using a rat cDNA clone (GGFRP3) that starts at the EGF domain and includes most of the cytoplasmic domain (see Fig. 1 in Chen et al., 1994: core 2 probe). Following dissection, retinas were fixed in 4% paraformaldehyde for 1-2 hours at room temperature or overnight at 4°C. Retinas were either sectioned on a cryostat as described above, or alternatively, dehydrated and embedded in Paraplast, prior to sectioning. The protocol for the in situ was basically the same for both the frozen and paraffin sections, although deparaffinization steps were added for the latter. All solutions were made with diethyl pyrocarbonate (DEPC)-treated water. The sections were treated with TEA and proteinase K prior to prehybridization in 50% formamide, 5× SSC, 5× Denhardt's, 250 μg/ml tRNA and 500 μg/ml salmon sperm DNA for 2-12 hours at room temperature. The sections were then hybridized in the same solution, but with probe added at 50-200 ng/ml at 72°C for at least 12 hours. The sections were rinsed with 5× SSC for 5 minutes at 72°C followed by 0.2× SSC for 1 hour at 72°C. Bound probe was visualized using an alkaline phosphatase-conjugated antibody to digoxigenin (Boehringer-Mannheim Biochemicals).

Polymerase chain reaction

Oligonucleotide primers were designed to the rat sequence of erbB2/neu (Bargman et al., 1986) and used to amplify retinal cDNA from newborn rat RNA. Newborn rat retinas were dissected free from the surrounding ocular tissues, rinsed in DEPC-treated PBS and frozen on dry ice. The tissue was then thawed and homogenized in lysis buffer containing 20 mM DTT and 750 U/ml RNasin in a 1.5 ml tube with a plastic pestle. Next, the lysate was under-lain with a 25% sucrose solution and centrifuged to pellet the unlysed cells and nuclei. The supernatant was treated with proteinase K at 37°C for 30 minutes, followed by two successive phenol/chloroform extractions and two chloroform extractions. Total RNA was then precipitated in ethanol, resuspended in Tris-EDTA (TE)+0.2% SDS, precipitated again in ethanol and 7.5 M ammonium acetate, then resuspended in TE and the concentration measured on a Beckman DU-70 spectrophotometer.

An aliquot of the total RNA was reverse transcribed with random hexamer primers for 1 hour at 42°C in a 20 μl reaction solution containing the following: 1-3 μg of retinal RNA, 50 mM KCl, 20 mM Tris-HCl pH 8.3, 2.5 mM $MgCl_2$, 0.001% gelatin, 1 mM dNTPs (Pharmacia, Pleasant Hill, CA), 30 units RNasin (Promega, Madison, WI), 100 pmol random hexamers (Pharmacia), 200-400 units reverse transcriptase MoMuLV (BRL, Bethesda, MD), and 10 mM DTT. The cDNA was boiled for 3 minutes, placed on ice for 3 minutes, then treated with 1 μl of 1 mg/ml RNase for >10 minutes at 37°C to remove any remaining RNA. An aliquot of the cDNA (10 μl) was then amplified for 30 cycles on a Coy thermocycler in a 50 μl reaction containing: 500 ng primers, 2.5 units Taq polymerase (BRL) and final buffer concentrations of 50 mM KCl, 10 mM Tris-HCl, pH 8.3 and 1.9-2.3 mM $MgCl_2$. The primer sequences used (upstream and downstream primers, respectively) for the erbB2/neu receptor tyrosine kinase domain amplification were bases 2683 to 2695 and 3151 to 3170. The PCR products were analyzed by electrophoresis on a 3% agarose gel to verify that the appropriate predicted sized product was produced. The resulting band was excised from the gel and ligated into pCR2 (Invitrogen) according to the manufacturer's protocol, and several clones were sequenced to confirm the identity of the product as erbb2/neu.

Cell culture

Following CO_2 overanaesthesia, the retinas from Long Evan's rats at embryonic days 15 and 18, and postnatal days 2, 5, and 10 were dissected free from scleral tissue and pigmented epithelium and dissociated for cell culture. The retinas were dissociated by incubation in calcium/magnesium free Hank's balanced salt solution (CMF-HBSS; GIBCO) containing between 0.1 and 0.25% trypsin (GIBCO) at 37°C with gentle rocking for 15 minutes. Trypsin was inactivated by adding fetal bovine serum (FBS; GIBCO) to the solution. Cells were then collected by centrifugation for 5 minutes at 1500 rpm, and the cells were resuspended in F12/Dulbecco's Modified Essential Medium (F12/DMEM, glutamate and aspartate free; GIBCO; as described by Reh, 1992) with the following serum supplements: insulin, transferrin, selenium, progesterone and putresceine. The cells were plated at a density of 30,000-40,000 cells/cm^2 on glass coverslips that had been previously coated with a fibrillar collagen gel (Vitrogen). Human recombinant GGF2 (rhGGF2) was added at the time of plating.

Fixation and immunostaining

Cell cultures were fixed overnight in 4% paraformaldehyde/4% sucrose at 4°C after 1 to 3 days in vitro. Different retinal phenotypes were identified by labelling cultures with the following cell specific antibodies.

1. Rat-401 (Nestin; from Dr S. Hockfield, Yale University School of Medicine, New Haven, CT) which labels intermediate filaments in progenitor cells (Cattaneo and McKay, 1990).

2. Anti-recoverin (from Dr Jim Hurley, HHMI, University of Washington, Seattle) which labels rod and cone photoreceptors and cone bipolars (Korf et al., 1992; Milam et al., 1993).

3. Anti-cellular retinoic acid binding protein (CRABP; from Dr J. Saari, University of Washington, Seattle) which labels amacrine cells in the rat retina (Gaur et al., 1990).

4. TUJ1 (from Dr A. Frankfurter, University of Virginia), an antibody directed against neuron-specific β-tubulin, which is present only in retinal ganglion cells early in embryonic development, but in postnatal retina is expressed by most retinal neurons.

5. RMO 160, an antibody (from Dr Virginia Lee) directed against the 160×10^3 M_r subunit of neurofilaments, which is only expressed in retinal ganglion cells and a small number of amacrine cells and horizontal cells in the rat retina (Drager et al., 1981).

6. GFAP, a polyclonal antiserum raised against bovine glial fibrillary acidic protein was obtained from Boehringer-Mannheim.

7. CRALBP, a rabbit polyclonal antiserum raised against the bovine cellular retinaldehyde binding protein, specifically identifies retinal Muller glia in the rat (also a gift from Dr J. Saari; De Leeuw et al., 1990).

For the immunohistochemistry of the cell cultures, the coverslips were incubated in the primary antibody overnight, followed by an appropriate (species) secondary antibody conjugated to either rhodamine, fluorescein (CAPPEL), or peroxidase (Vector Labs) to visualize immunoreactivity.

Analysis of cultures

To quantify the effects of neuregulin on cell survival, we used three different methods of calculating cell number. For most experiments, we counted the cells in a vertical strip across the length of each coverslip using the 40× objective on the Zeiss compound microscope. In other experiments, we counted the number of neurons and those with neurites greater than one cell diameter in six randomly selected microscope fields with the 40× objective. In those experiments where the cell density after 2 days in vitro was very low (i.e. the late postnatal cell cultures), the total number of cells on the coverslip (and those with processes) were counted. The number of repetitions and the statistical analysis of the data is detailed in the figure legends for each experiment.

Analysis of apoptosis in vitro

We determined the number of apoptotic cells in the cultures with the TUNEL method using terminal transferase to incorporate digoxigenin-labelled nucleotides into the DNA of dying cells (Apoptag kit from Oncor). The labelling was carried out according to the directions of the manufacturer. The number of labelled cells for each experimental condition was determined in six randomly selected fields at 40× magnification.

RESULTS

Neuregulin mRNA and protein are present in the developing retina

A previous study (Meyer and Birchmeier, 1994) has shown that neuregulin mRNA is expressed in the retinal ganglion cell layer of the embryonic mouse. We used digoxigenin-labelled sense and antisense RNA probes to analyze the expression of neuregulins in developing rat retinas. The probe used extended from the EGF-domain through the cytoplasmic domain, and thus should hybridize with all known forms of neuregulin transcripts. Fig. 1A-C shows the distribution of mRNA for neuregulin in E18, P0 and P15 retinas, respectively, while Fig. 1D-F shows the sense strand controls. At E18, there is a low level of hybridization of the antisense strand throughout the retina (Fig. 1A) when compared to the sense strand control (Fig. 1D). In the neonatal rat retina (P0) there is an overall increase in the level of neuregulin expression, and now the labelling of the cells in the ganglion cell layer can be more clearly seen (Fig. 1B, arrows). By postnatal day 15, when the retina has acheived its mature structure, the message for neuregulin is more concentrated in the ganglion cell and inner nuclear layers; however, there is also a lower level of neuregulin expression in the outer nuclear layer (ONL). These results extend those of Meyer and Birchmeier (1994) to show expression of neuregulin in retinal cells throughout retinal development and in mature retina.

To confirm that the mRNA is translated and neuregulin protein is produced by retinal cells during development, we used both western blotting and immunohistochemistry. We used three different antibodies directed against different

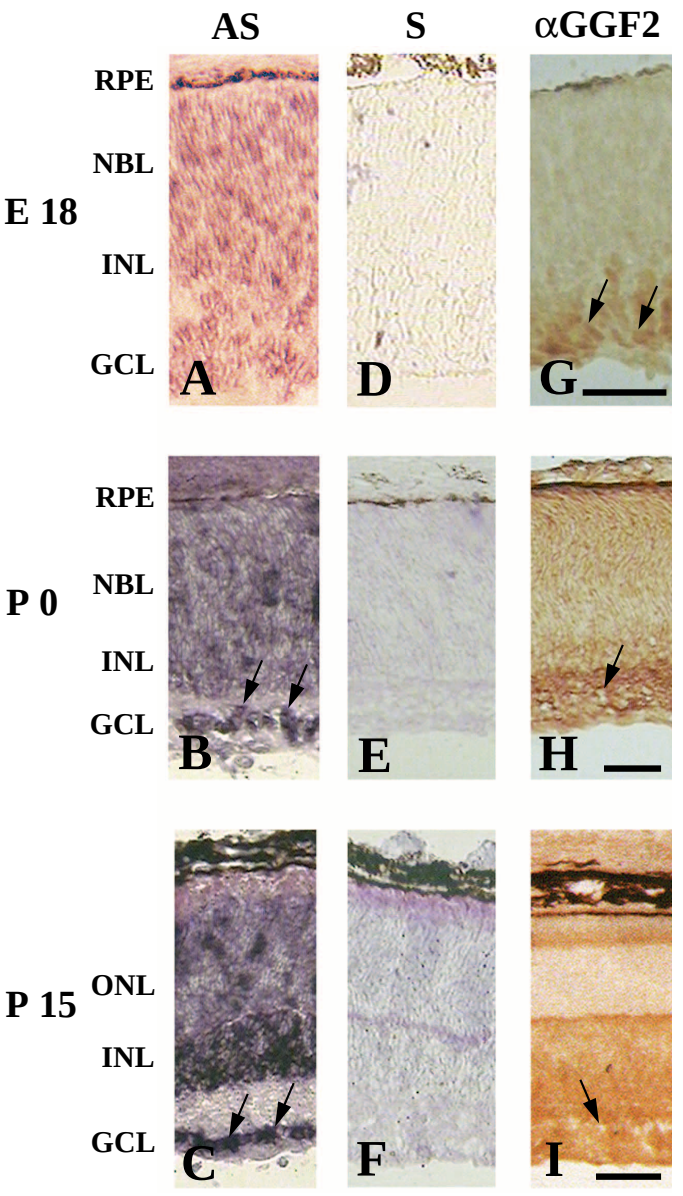

Fig. 1. In situ hybridization and immunohistochemistry show GGF/neuregulin expression in embryonic and postnatal rat retina. Photomicrographs of in situ hybridization with antisense (A-C) and sense (D-F) digoxigenin-labelled probes for neuregulin in embryonic day 18 (A,D), postnatal day 0 (B,E) and mature (C,F) rat retina. Label is concentrated in the ganglion cell layer (arrows) in the neonatal and mature retina. G,H, and I show immunohistochemistry of embryonic, postnatal and mature rat retina using CN16 antibody. Scale bar, 50 μm.

epitopes of the molecule (see Materials and Methods) and they all gave similar immunohistochemical results. For the western blots, we probed retinal and brain homogenates with the CN16 antibody raised against rhGGF2. The results are shown in Fig. 2C, lanes 1 and 2 for postnatal day 15 rat retina and brain, respectively. We observed several distinct bands in both retina and brain. Immunoreactive proteins with approximate relative molecular masses (M_r) of 100×10^3, 80×10^3 and 49×10^3 were present in both retina and brain, although the band of 80×10^3 M_r was very faint in the retina. The brain also had a very high relative molecular mass immunoreactive protein, migrating at approximately 180×10^3, though this was not present in the retina. Alternatively, in the retina, there was a band of approximately 60×10^3 M_r that was not present in the brain homogenate. With the exception of the bands of 180×10^3 and the 80×10^3 M_r, these molecular masses are approximately the same as those observed by Sandrock et al. (1995), with antibodies directed against ARIA, and are likely to represent polypeptides coded from alternately spliced products of the neuregulin gene. However, 80×10^3 is the relative molecular mass of rhGGF2 and so it is likely that this band also represents a form of neuregulin. All of these bands were no longer present in blots incubated with antiserum that had been preabsorbed with 16 molar excess rhGGF2.

Fig. 1G-I shows the results of the immunohistochemistry with the same antibody against rhGGF2. Neuregulin antibodies show specific immunoreactivity in the retinal ganglion cell layer at E18 and postnatal day 1 (Fig. 1G,H) and this immunoreactivity can be blocked by preabsorption of the antiserum with rhGGF2 (Fig. 4A,B). This concentration of neuregulin immunoreactivity in the ganglion cell layer at these stages is in contrast to the more widespread distribution of the mRNA (compare Fig. 1B and 1H). This difference is likely due to the fact that the probe used will hybridize with all known forms of neuregulin, while the antibody may only recognize a subset of neuregulin isoforms. At P0, neuregulin immunoreactivity is present in the developing inner plexiform layer, consistent with a recent report showing some neuregulin isoforms in synaptic zones in the brain and spinal cord (Sandrock, 1995); however, there is no message expression in the inner plexiform layer, since this synaptic zone is free from cell bodies. In the mature retina, neuregulin immunoreactivity is present in the synaptic zone of the inner plexiform layer and in the cells of the ganglion cell layer and the cells of the inner nuclear layer. While photoreceptor cell bodies do not show immunoreactivity their inner segments do show a low level of immunoreactivity (Fig. 1I, arrowheads). These results confirm that neuregulin proteins are present in the developing and mature rat retina. As observed for the mRNA for neuregulin, the immunoreactivity is more highly concentrated in the inner retina than the outer nuclear layer cells (photoreceptors). It is also of interest that the photoreceptor cell inner segments are immunoreactive for neuregulin, since this same region shows distinct erbB3 immunoreactivity (see below).

Neuregulin receptors are expressed in the retina during embryonic and postnatal development

The putative receptors for the neuregulins are members of the EGFR family of receptor tyrosine kinases, erbB2/neu, erbB3 and erbB4/tyro2. These proteins all bind neuregulins with as homo- or heterodimers (Carraway et al., 1994; Lupu et al., 1990; Peles et al., 1993; Plowman et al., 1993; Kita et al., 1994; Kraus et al., 1993; see Carraway and Cantley, 1994, for review). Therefore, we used immunohistochemistry, western blotting and PCR to determine: (1) whether these receptors are expressed in retina; (2) whether these receptors can be phosphorylated in response to rhGGF2; and (3) the cellular distribution of the three neuregulin receptors during retinal development.

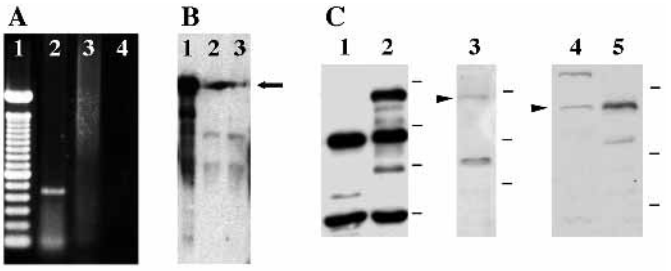

Fig. 2. Neuregulin and the erbB family of receptors are expressed in rat retina. (A) The results of RT-PCR for erbB2/neu in the developing rat retina. Lane 1 shows the molecular mass markers, lane 2 shows that a 200 bp product can be amplified from P0 rat retinal cDNA, but is not present when the reverse transcriptase or template are omitted (lanes 3 and 4 respectively). (B) A phosphotyrosine blot of cultured newborn rat retinal cells treated with rhGGF2 (25ng/ml; lane 2) or no treatment (lane 3). Lane 1 is an extract of 431 cells treated with EGF and shows phosphorylation of the EGF receptor and serves as a positive control for the phosphotyrosine antibody. (C) Western blots for GGF and erbB receptors. Lanes 1 and 2 show proteins from P15 retina and brain probed with CN16, a polyclonal rabbit antibody to rhGGF2. Molecular mass markers are indicated at the right of the blot and from the top are 200×10^3, 116×10^3, $80\times10^3$and 49.5×10^3. Lane 3 shows a retinal extract probed with erbB3 antibody and lanes 4 and 5 show retina and brain extracts probed with erbB4 antibody. Molecular mass markers are indicated at the right of the blot and from the top are 200×10^3, 116×10^3, and 80×10^3.

erbB2/neu

To determine whether erbB2/neu was expressed during retinal development, we used RT-PCR with retinal RNA isolated from newborn rat pups. With primers specific for erbB2/neu, we were able to amplify a distinct band of the appropriate molecular mass(Fig. 2A, lane 2) and subsequent cloning and sequencing showed this to be 98% identical to the rat erbB2/neu sequence in GenBank. We used immunohistochemistry with three different erbB2/neu antibodies to determine which cells express erbB2/neu in embryonic, neonatal and adult rat retinas. Two of the antibodies, 96G and AB4, which were directed against two distinct epitopes in erbB2/neu have been previously used to study the expression of erbB2/neu in neuronal cells (Schwechheimer et al., 1994; Shah et al., 1994). These gave identical patterns of immunoreactivity in the retina. Thus, these two were used to characterize the distribution of this receptor. A third antibody, (K-15) directed against peptide sequences in the conserved kinase domain gave a much more widespread pattern of immunoreactivity; it is likely that this antibody recognizes other EGFR-related kinases as well erbB2/neu and so its pattern of immunoreactivity will not be described in detail.

The neu/erbB2 immunoreactivity was developmentally regulated in the embryonic rat retina. In embryonic day 15 rat retina, we did not observe any immunoreactivity for neu/erbB2 (data not shown); however, in embryonic day 18 and 19 retina, distinct erbB2/neu immunoreactivity is present in the developing inner plexiform layer, between the ganglion cell layer and the differentiating inner nuclear layer cells (Fig. 3A, arrows). Both of the two erbB2/neu antibodies label the neuronal processes in the plexiform layer, which at this time consists of the dendrites of ganglion cells and amacrine cells,

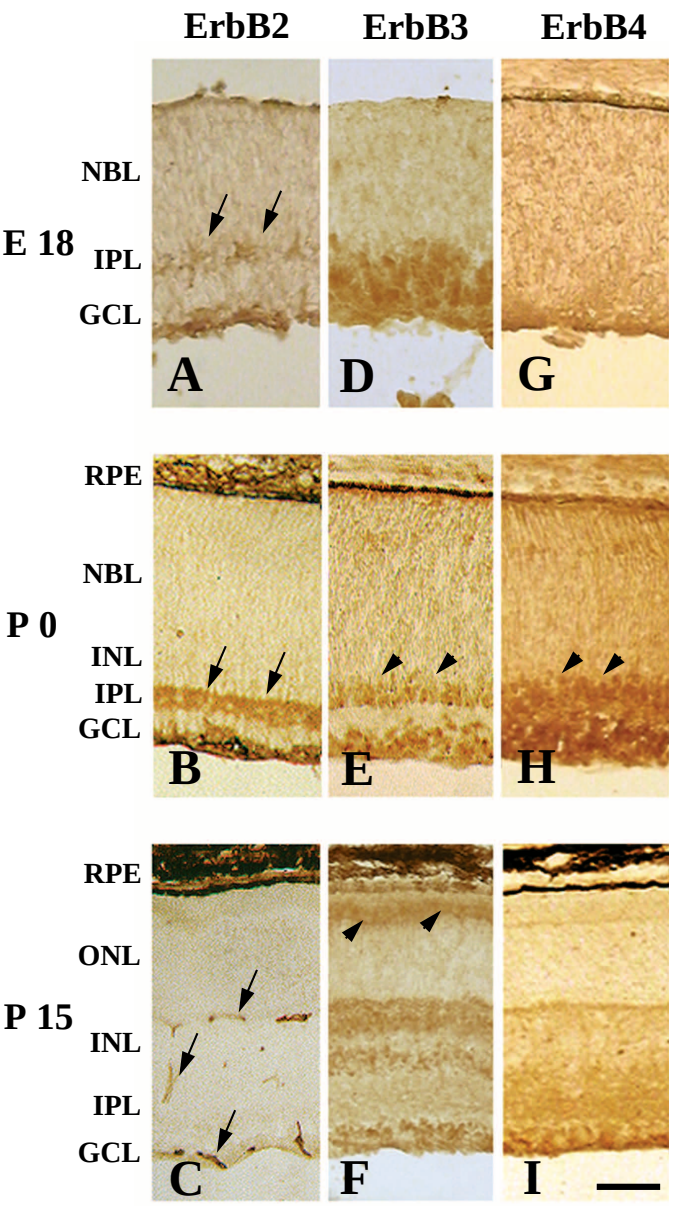

Fig. 3. Receptors for neuregulins are expressed in the developing and mature rat retina. Photomicrographs of cryostat sections incubated with antibodies for neu/erbB2 (A-C), erbB3 (D-F) and erbB4/tyro2 (G-I) in E18 (A,D,G), newborn (B,D,H) and P15 (C,F,I) rat retinas. While all of the receptors show a changing developmental pattern, erbB2/neu and erbB3 are expressed in the E18 retina, all three receptors are expressed in the P0 retina, and erbB3 and erbB4/tyro2 are expressed in the mature (P15) retina. Scale bar, 50 μm.

since the retinal bipolar cells are not generated until after birth in rodents (Young, 1983; Sidman, 1961).

There is also expression of erbB2/neu in the neonatal retina. By the first postnatal day, the immunoreactivity for erbB2/neu in the inner plexiform layer has increased markedly over that seen in the embryonic retina (Fig. 3B, arrows). A similar pattern of immunoreactivity is observed in retinas from postnatal day 2 and postnatal day 5 animals. This expression of erbB2/neu is transient, however, and by postnatal day 15, it has disappeared (Fig. 3C). In the mature retina, there is no

more immunoreactivity for erbB2/neu in the neural retinal cells; however, the vascular endothelial cells, which have now invaded the retinal parenchyma, are highly immunoreactive (Fig. 3C, arrows).

erbB3

To determine whether erbB3 is expressed in the developing retina, we used western blots with an antibody directed against a peptide in erbB3 (see Materiasls and Methods). This antibody is known not to cross-react with neu/erbB2 or erbB4 (Jo et al., 1995; Graus-Porta et al., 1995). Fig. 2C, lane 3 shows that a polypeptide of approximate molecular mass 180×10^3 is present in a membrane preparation from postnatal day 15 rat retinas (arrowhead). An additional doublet of bands migrating at approximately 100×10^3 M_r is also observed in the retinal membranes and is presumed to be a truncated form of the receptor. When this antibody is used in immunohistochemistry on retinal sections, the pattern of immunoreactivity for erbB3 is distinctly different from erbB2/neu; erbB3 is present almost exclusively in cells in the retinal ganglion cell layer in the embryonic retina (Fig. 3D). In the neonatal retina, in addition to the retinal ganglion cells, some cells in the inner nuclear layer are also immunoreactive (Fig. 3E, arrowheads); however, in contrast to erbB2/neu, there is no label in the inner plexiform layer. This immunoreactivity is eliminated by preabsorption with the immunizing peptide (Fig. 4C,D). By postnatal day 15, virtually all of the neural retinal cells show some level of expression of erbB3. Most retinal ganglion cells are labelled, as are amacrine cells, bipolar cells, the inner and outer plexiform layers and the inner segments of the photoreceptors (Fig. 3F). Thus, erbB3 has a much more widespread distribution than erbB2/neu, and has a very different developmental regulation. Nevertheless, erbB3 is expressed in retinal ganglion cells and amacrine cells during the same period that erbB2/neu immunoreactivity is present in their processes in the inner plexiform layer; therefore, it is possible that erbB2/neu acts as a co-receptor with erbB3 for neuregulin signalling in these cells.

erbB4/tyro2

The last of the putative neuregulin receptors that we analyzed is erbB4/tyro2; we used an antibody directed against a carboxy-terminal peptide specific for this receptor, and not present in the related erbB receptors. Western blots with retinal and brain membrane preparations show a distinct band of M_r 180×10^3 in both tissues (Fig. 2C, lanes 4 and 5, respectively, arrowhead). In the retina, there is also a very high molecular mass protein which may be related to a recently described novel EGFR-related tyrosine kinase isolated from human gliomas (Panneerselvam et al., 1995). There is a low level of immunoreactivity for erbB4/tyro2 in the ganglion cell layer of the E18 retina (Fig. 3G). In the early postnatal retina, erbB4/tyro2 is present in neurons in the ganglion cell layer and the inner half of the inner nuclear layer, as well as in the processes of the ganglion cells and amacrine cells in the inner plexiform layer (Fig. 3H). All three erbB4 antibodies showed a similar pattern of immunoreactivity, although the two mouse monoclonal antibodies showed a less intense staining in the INL than that shown in the figure for the rabbit antiserum. In the mature retina, the erbB4/tyro2 immunoreactivity is similar to that of erbB3; most of the neurons of the retina, with the exception of the photoreceptors, are labelled with the erbB4/tyro2 antibody (Fig. 3I, ONL). Thus, the pattern of expression of erbB4 overlaps with both erbB3 and erbB2/neu in the developing retina. This similar pattern of expression suggests that erbB4/tyro2 might also act as a co-receptor for erbB3 and erbB2/neu in the developing retina; however, in the mature retina, when erbB2/neu is no longer expressed, erbB4/tyro2 is more likely to interact with erbB3. While most neurons in the mature retina express both erbB3 and erbB4/tyro2, the photoreceptors express only erbB3.

In sum, all three putative receptors for neuregulins are expressed in the developing retina, and both erbB3 and erbB4/tyro2 are also expressed in adult retina. Since several different classes of retinal neurons express receptors for the neuregulins in both the developing and mature retina, it is possible that neuregulins have direct actions on retinal neurons. To determine whether these receptors can be phosphorylated in response to rhGGF2, we dissociated cells from newborn rat retinas and after 24 hours added fresh serum-free medium with or without 20 ng/ml rhGGF2 and continued the incubation for 2.5 minutes. The medium was then removed and the cellular proteins were solubilized with NP-40 lysis buffer containing 1 mM sodium orthovanadate and protease inhibitors (see Materials and Methods). The proteins were separated by SDS-PAGE, blotted onto nitrocellulose and probed with an antibody

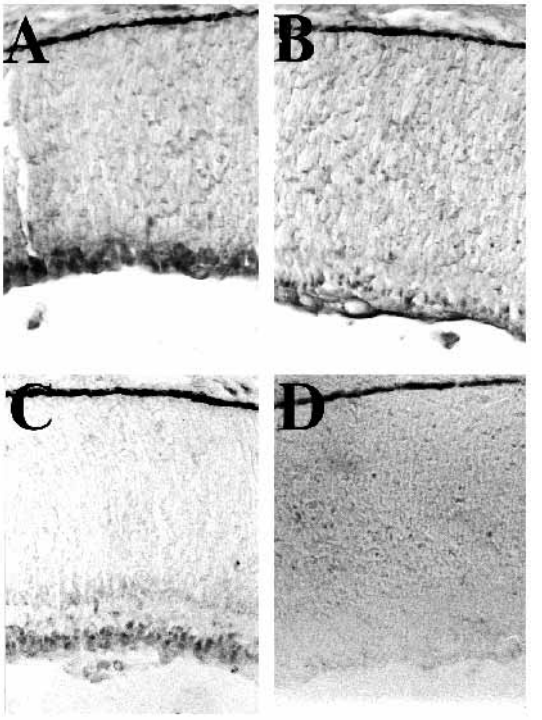

Fig. 4. Neuregulin and erbB3 immunoreactivity can be blocked by preabsorption with the antigens. A shows a micrograph of a P1 rat retinal section incubated with the CN16 antibody raised against rhGGF2, while B shows a similar section processed identically, but with antibody that had been preabsorbed with a 16-fold molar excess of rhGGF2. The immunoreactivity in the ganglion cell layer is eliminated by the antigen. C shows a section from P1 rat retina incubated with the erbB3 antibody, and D shows a similar section processed identically, but with an antibody that had been preabsorbed with 100-fold excess of the immunizing peptide.

raised against phosphotyrosine (Fig. 2B). Lane 1 is a positive control, showing EGFR phosphorylation in response to EGF treatment of 431 cells. Lane 2 shows phosphorylation of a 170-180×10^3 M_r band (arrow) in the rhGGF2 treated cells while lane 3 shows the lower level of phosphorylation observed in the untreated retinal cells.

GGF promotes neuronal survival and neurite outgrowth in serum-free cultures of rat retina

To test for possible actions of neuregulins on retinal development, we cultured retinal cells in the presence of various concentrations of rhGGF2 and assayed for effects on cell survival, and neurite growth. The effects of rhGGF2 on retinal neuronal survival and neurite extension were assayed in embryonic and neonatal retinal cells cultured at low density in collagen gels. Seeding the cells in collagen gels blocks their mitotic activity and forces progenitor cells to differentiate prematurely (Reh and Kljavin, 1989). When this is done at embryonic stages of development, many of the differentiated neurons in the resulting cultures are ganglion cells, and the collagen gel supports their neurite extension (Kljavin and Reh, 1991). rhGGF2 was added to these cultures while sister wells with no added factors served as controls. The rhGGF2 preparation we used is >95% pure, and is a glycosylated protein with an average molecular mass of 80×10^3 by SDS-PAGE. We chose a concentration range of 1-500 ng/ml or 0.01-6.25 nM, similar to that used in previous studies of rhGGF2. Previously it had been shown that rhGGF2 has effects on neural crest stem cell differentiation and as a mitogen for Schwann cells at concentrations of 0.1-10 nM (Shah et al., 1994) and activation of acetylcholine-receptor gene expression occurs at 5 nM (Jo et al., 1995). After culture periods of from 1 to 3 days, the cells were fixed in 4% paraformaldehyde and immunohistochemical analysis was carried out using several different antibodies to characterize the effects of the factor.

In an initial series of experiments, we quantified the effects of rhGGF2 on the total number of cells surviving in the culture after 2 days, using phase microscopy. We plated on average 30,000-40,000 cells/cm^2. In serum-free medium, on average, approximately 68% of the cells that are initially plated die within the first 2 days of culture. Addition of rhGGF2 to the wells prevented this cell loss in a dose-dependent manner (Fig. 5A). Although the effect was small at 1 ng/ml and 10 ng/ml, at 100 ng/ml (approximately 1.25 nM), there were over twice as many cells surviving in the rhGGF2-treated wells as in the controls. Thus, when a saturating concentration of rhGGF2 is added to dissociated retinal cell cultures, a majority of the cells now survive for 2 days.

To determine whether the surviving cells in the rhGGF2-treated wells were neurons, we labelled the cultures with an antibody against neuron-specific β-tubulin, TUJ1. In sections of embryonic retina, TUJ1 labels cells in the ganglion cell layer and the inner half of the developing inner nuclear layer, but as development proceeds nearly all the cells in the inner retina express this antigen. The majority of the cells in the cultures of embryonic retina were TUJ1 immunoreactive after 2 days in vitro. We counted the number of TUJ1 immunoreactive neurons after two days in the cultures (Fig. 6A-C). We found a dose dependent increase in the number of neurons in the rhGGF2-treated wells (Fig. 5B). At the maximum concentration of the factor, the rhGGF2-treated cultures had approximately three times as many TUJ1 immunoreactive neurons as in the control wells. This increase in TUJ1 immunoreactive neurons with the addition of rhGGF2 could be due to the promotion of survival of differentiating retinal neurons, or alternatively rhGGF2 may be promoting the neuronal differentiation of retinal progenitor cells. The latter possibility is unlikely, since as noted above, retinal progenitor cells isolated in low density collagen substrates rapidly differentiate into the various types of retinal neurons in our medium (Reh and Kljavin, 1989). Moreover, immunohistochemistry for the GGF receptors, erbB2, erbB3 and erbB4 failed to show any immunoreactivity in the progenitor zone of the developing retina. Nevertheless, to directly assay for potential survival effects of rhGGF2 on retinal cells, we used an assay for apoptosis.

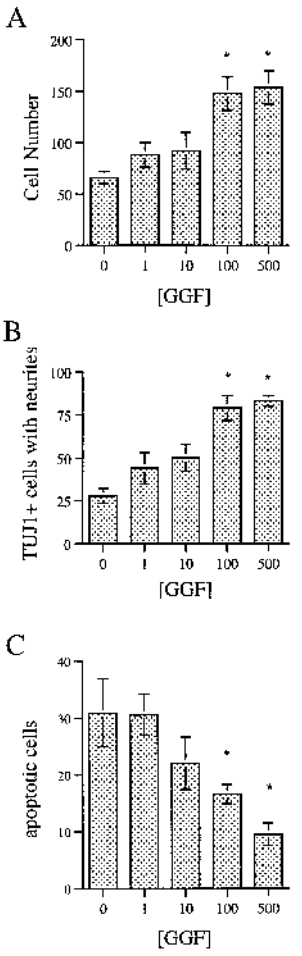

Fig. 5. GGF has direct actions on retinal neurons in low density culture. Addition of rhGGF2 to retinal cultures promotes cell survival (A), promotes neurite outgrowth (B) and reduces apoptotic cell death (C) in a dose-dependent manner. The graphs all show the means and standard errors for a minimum of three experiments in which rhGGF2 was added to the medium at various concentrations. Concentrations of rhGGF2 are given in ng/ml. Mean values statistically different from control values (P<0.001) are designated with an asterisk (Student's t-test).

The majority of the retinal cells that die in the first 2 days of culture undergo an apoptotic cell death (Reh, unpublished observations). Therefore, to further analyze the survival effects

of rhGGF, we used a terminal transferase labelling reaction (TUNEL) to compare the number of apoptotic cells in rhGGF2-treated and control wells. Fig. 6D,E shows examples of TUNEL-labelled cells after 2 days in vitro, and Fig. 5C is a graph of the number of apoptotic cells as a function of rhGGF2 concentration. There was a dose-dependent decrease in the number of apoptotic cells in the rhGGF2-treated wells, consistent with the hypothesis that this molecule can act as a survival factor for some retinal neurons in vitro.

We also observed that the neurons in the rhGGF2-treated wells had a greater number of neurites per cell on average. Fig. 6A,C shows examples of TUJ1 immunoreactive cells from E18 retinas after 2 days in vitro from rhGGF2-treated cultures; Fig. 6B shows cell from control wells. Neurons in the rhGGF2-treated wells typically had more extensive neurite outgrowth, with more complex arborizations. The average number of neurites longer than one cell diameter per cell is plotted as a function of rhGGF2 concentration in Fig. 7A. The data show that rhGGF2 causes the TUJ1 immunoreactive cells to extend a greater number of neurites in vitro. However, the average length of the longest neurite was not significantly different with the addition of rhGGF2; the average length of the longest neurite in the 100 ng/ml and 500 ng/ml rhGGF2 concentrations were 112% and 91% of control (no statistically significant differences were observed between groups when tested with a

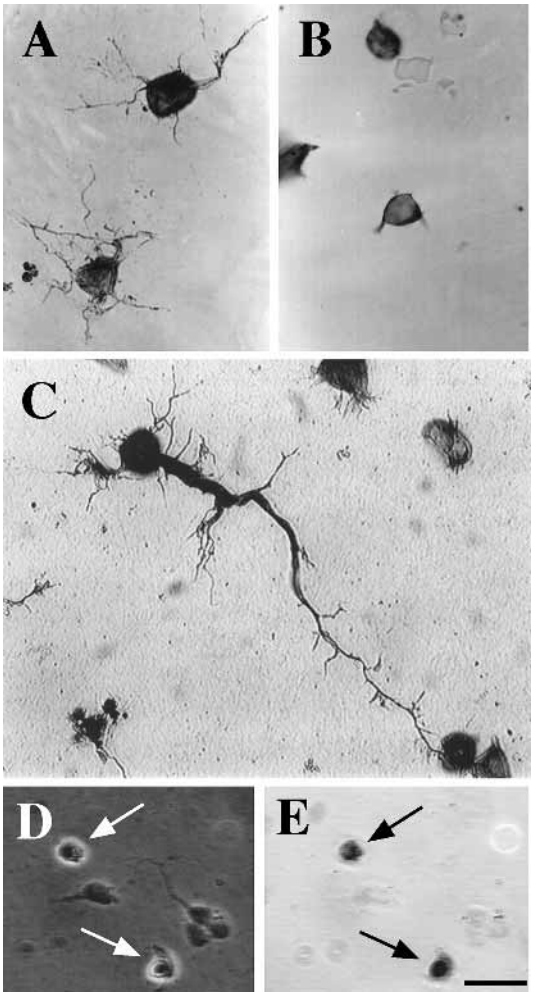

Fig. 6. rhGGF2 promotes cell survival and neurite outgrowth of TUJ1 immunoreactive cells. A and C show examples of rhGGF2 (100 ng/ml) treated E18 retinal TUJ1 immunoreactive neurons after 2 days in vitro, demonstrating the extensive neurite outgrowth promoted by this factor. Control TUJ1 immunoreactive neurons are shown in B. These cells have fewer neurites per cells, and less branched neurites. D and E show corresponding phase contrast and bright-field photomicrographs of E18 retinal cells labelled for apoptotic cells using the TUNEL method. Arrows point to the same cells in both panels. Scale bar, 10 μm (A-C) and 20 μm (D,E).

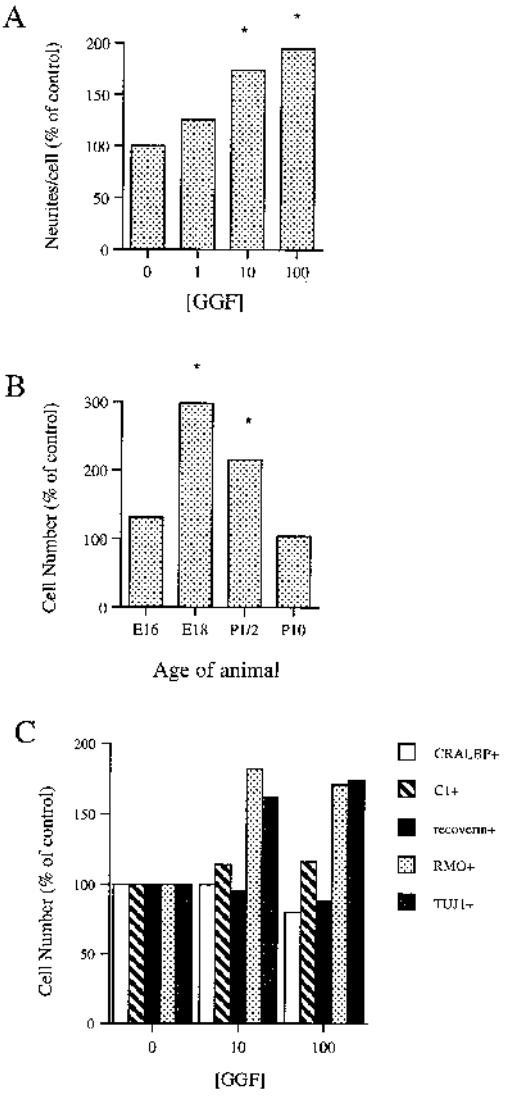

Fig. 7. rhGGF2 promotes neurite outgrowth and survival in an age-dependent manner and its effects are specific for retinal ganglion cells. A shows the number of neurites per cell as a percentage of the control value as a function of rhGGF2 concentration for embryonic day 18 TUJ1 immunoreactive neurons. B shows the number of TUJ1 immunoreactive cells with neurites longer than one cell diameter, for four different ages of retinal cultures, to show the age dependence of the response. Mean values statistically different from control values ($P<0.001$) are designated with an asterisk (Student's *t*-test). C is a graph of the number of several different retinal cell types as a function of rhGGF2 concentration (ng/ml) in neonatal retinal cells after 2 days in vitro. The values for RMO and TUJ1 immunoreactive cells in the GGF-treated wells were significantly different from control wells ($P<0.01$) using a Student's *t*-test.

non-paired Student's *t*-test). Thus, rhGGF2 causes the initiation or maintenance of neurites in retinal neurons, but does not apparently promote the further extension of these processes.

The response to GGF is developmentally regulated

Since neuregulin receptors show developmental changes in their expression during retinal development (see above), we were interested to know whether there might be some change in the response to rhGGF2 during this period. Therefore we also examined the effect of rhGGF2 on embryonic day 16, postnatal day 1 or 2 and postnatal day 10 retinal cell cultures. Cells from retinas of embryonic day 16 and postnatal days 1or 2 and 10 were plated on collagen gels in serum-free medium at approximately the same density as that for the E18 cultures described above. rhGGF2 was added at a final concentration of 1-500 ng/ml. When the embryonic day 16 or postnatal day 10 cultures were analyzed after 2 days in vitro, there was no significant increase in the number of retinal neurons with processes for any of the rhGGF2 concentrations we used (Fig. 7B). By contrast, the postnatal day 2 cells responded to rhGGF2 in much the same way as the embryonic day 18 cells; there was a significant increase in the number of TUJ1 immunoreactive neurons in the neonatal cultures treated with rhGGF2 (Fig. 7B).

GGF acts on retinal ganglion cells

The above results demonstrate an action of rhGGF2 on retinal neurons. While TUJ1 is expressed in retinal ganglion cells during early embryonic stages, other types of retinal cells are also TUJ1 immunoreactive. Therefore, we used additional neuron-specific antibodies to determine which types of retinal neurons were affected by this factor. We assayed for the effects of rhGGF2 using an additional ganglion cell marker, an antibody directed against the 160×10^3 M_r neurofilament protein and antibodies specific for two other classes of retinal cells, amacrine cells and photoreceptors. A monoclonal antibody raised against cellular retinoic acid binding protein (or C1) labels a large number of the amacrine cells in the rat retina. C1 immunoreactivity is present in large multipolar neurons in both the rhGGF2-treated and control cultures after 1 or 2 days in vitro. A polyclonal antiserum raised against recoverin, a protein present in both classes of photoreceptors – rods and cones – as well as cone bipolar cells, was used to identify these cell types in the cultures.

Amacrine and photoreceptor cell survival and neurite extension do not appear to be influenced by rhGGF2. Nearly all (98%) of the C1 immunoreactive cells had multiple neurites after 1 and 2 days in vitro, in both the control and GGF-treated cultures. There was no significant effect of rhGGF2 on the total number of C1 immunoreactive cells (Fig. 7C). In addition, there was no change in the number of recoverin immunoreactive cells (Fig. 7C); in both the rhGGF2-treated and the control wells, recoverin immunoreactive cells had few or no neurites, typical of photoreceptors when grown in collagen gels (Kljavin and Reh, 1991). Cells labelled with an additional antibody marker for retinal ganglion cells, anti-neurofilament (RMO), show the same response to rhGGF2 as the TUJ1 immunoreactive cells. In the neonatal cultures, rhGGF2 causes an increase of between 150% and 200% in the cells with neurites that are immunoreactive for RMO and TUJ1 (Fig. 7C).

We also examined whether rhGGF2 promotes retinal glial, differentiation or survival, as has been reported previously for other regions of the nervous system. Two antibody markers were used in our experiments; CRALBP was used to label the intrinsic retinal glia, the Muller cells, while GFAP was used to identify astrocytes that have migrated into the retina from the optic nerve. In general our culture conditions – low density plating on collagen gels without serum – do not support glial proliferation. The percentage of Muller glia in the cultures ranged from 3.5-5% in dissociated cell cultures of postnatal day 2 retina after 2 days in vitro, and did not increase with the addition of rhGGF2 to the cultures (Fig. 7C). The number of GFAP immunoreactive astrocytes was less than one percent of the total cells for all conditions (data not shown). These results suggest that the effects of rhGGF2 on retinal neurons that we have observed are not due to an indirect action on the promotion of retinal glial proliferation, survival or differentiation; however, it is possible that under conditions more favorable to glial growth, rhGGF2 may have effects on Muller cells and retinal astrocytes.

DISCUSSION

The results of this study show the following: (1) some form of neuregulin is expressed in the embryonic and adult rat retina; (2) receptors for neuregulins are expressed in a developmentally regulated manner by several classes of retinal neurons; (3) neuregulins have direct effects on neurons in the developing CNS; and (4) neuregulins promote neuronal survival and neurite outgrowth.

Neuregulins are expressed in the embryonic and adult retina

Our results confirm and extend the previous study by Meyer and Birchmeier (1994), which demonstrated neuregulin expression in the retinal ganglion cell layer of the day 18 embryonic mouse. We found that the message for at least one form of this factor is expressed in the cells of the retinal ganglion cell layer in both the developing and mature retina. We also used immunohistochemistry to show that the neuregulin message is translated, and the protein is present in the retinal ganglion cell layer in the developing rat retina. Thus, in addition to proposed intraretinal functions of neuregulins (see below), their distribution is also consistent with a potential function analogous to that in the neuromuscular system; i.e. retinal ganglion cells may transport neuregulins to their targets to regulate neurotransmitter receptor synthesis.

In addition to the retinal ganglion cells, neuregulin is also expressed by other neurons in the retina. In the mature retina, neuregulin mRNA and immunoreactivity are present not only in retinal ganglion cells, but also in neurons in the inner nuclear layer, the amacrine cells, bipolar cells and horizontal cells; while photoreceptor cells have a much lower level of neuregulin mRNA expression than the other neurons, the photoreceptor inner segments show distinct neuregulin immunoreactivity. Although we have not been able to demonstrate any effect of neuregulin in our cultures after the neonatal period, the continued expression of the factor suggests that it regulates some other parameters of retinal biology that we failed to assay.

Neuregulin receptors are expressed by retinal neurons in a developmentally regulated manner

We have found evidence for the presence of all three of the known receptors for neuregulins in the retina during development; neu/erbB2, erbB3/HER3 and erbB4/HER4/tyro2 are all expressed by retinal neurons, particularly the ganglion cells and amacrine cells. These receptor tyrosine kinases have all been shown to bind neuregulins as homo- or hetero-dimers, leading to their phosphorylation (Carraway et al., 1994; Lupu et al., 1990; Peles et al., 1993; Plowman et al., 1993). We have also found that a p185 protein is rapidly and specifically phosphorylated in response to the addition of rhGGF2 to the medium of cultured retinal cells (data not shown).

Our data raise the possibility that developmental changes in receptor heterodimer combinations may regulate different functions of neuregulins. ErbB2/neu is specifically expressed in the developing inner plexiform layer of the retina in a very restricted period during late embryonic and neonatal development. By contrast, erbB3 and erbB4 are expressed in the same cells throughout most of development. Previous studies have suggested that these receptors normally act as heterodimers (for review, see Carraway and Cantley, 1994). For example, erbB2/neu may require the presence of one of the other receptors in order to bind neuregulin (Peles et al., 1993; Sliwkowski et al., 1994). Moreover, erbB3 has changes in the conserved kinase domain that are thought to result in a reduced autophosphorylation following neuregulin binding; however, cells expressing both erbB3 and erbB2 show a high level of phosphorylation of both molecules upon treatment with neuregulin (Sliwkowski et al., 1994; Carraway et al., 1994; Marikovsky et al., 1995). Thus, in the retina, it may be that erbB2/neu expression confers upon the retinal ganglion cells a particular sensitivity to neuregulin over a tightly regulated developmental period. The transient expression of erbB2/neu that we have observed in the developing retina may be a common feature of developing neuropil in other areas of the CNS, since transient erbB2/neu expression has also been reported in embryonic brain (Kokai et al., 1987).

Neuregulins have direct effects on neurons in the developing central nervous system

We have shown in this study that neuregulins have direct effects on neurons in the developing CNS. Using low density collagen gel cell cultures, where neurons are grown in effective isolation, we have found that rhGGF2 promotes neurite extension/initiation in a dose-dependent manner. It is unlikely that the effects we have observed in retinal neurons are mediated by glial cells in the cultures for three reasons. First, at the earliest time points at which we have cultured the retinal cells, Muller glial cells have not yet been generated (see Reh, 1991, for review), and the retinal astrocytes have not yet migrated into the retina from the optic nerve (Watanabe and Raff, 1992). Second, we have analyzed our cultures for effects on glial cell differentiation with several different glial markers, and we have not found any effects of neuregulin on glial cell number or differentiation under our culture conditions. Third, our immunohistochemical results for the neuregulin receptors erbB2/neu, erbB3 and erbB4/tyro2 show them to be expressed by neurons and the immunoreactivity for all of these proteins is present in the retina prior to the genesis of retinal glia (see Reh, 1991 for review).

Previous studies of the actions of neuregulins have concentrated on their effects on glial cells. One of the first known activities for this class of factors was its ability to stimulate the proliferation of Schwann cells (Brockes, 1987). Neuregulin has also been shown to promote the survival of astrocytes and the differentiation of oligodendrocytes in the CNS (Pinkas-Kramarski et al., 1994; Vartanian et al., 1994). During the development of the neural crest, neuregulin suppresses neuronal differentiation in vitro (Shah et al., 1994). However, recent studies have also shown effects of neuregulin on neurite outgrowth in PC12 cells (Corfas and Fischbach, personal communication) and neuronal channel expression (Role, personal communication).

Neuregulins promote retinal ganglion cell survival and neurite outgrowth

Our results also demonstrate novel activities for neuregulin. We have found that this factor promotes survival and neurite outgrowth from retinal neurons in vitro. Our finding that erbB2/neu is expressed in the developing retinal synaptic plexiform layers, in correlation with the normal process of cell death and dendritic elaboration of the retinal ganglion cells (see below), supports the possibility that neuregulin normally acts on these cells in vivo.

Neuregulins may normally be important regulators of ganglion cell number during development. Rat retinal ganglion cells establish synaptic connections with their central targets, the lateral geniculate nucleus and the superior colliculus, in the late embryonic period, presaging the period of naturally occurring retinal ganglion cell death in the first postnatal week (see Beazley et al., 1987 for review). During this period the number of retinal ganglion cells declines by about half of that initially generated. Since neuregulin receptors, including erbB2/neu, erbB3 and erbB4 are all expressed in retinal ganglion cells during the late embryonic and neonatal period, there is a temporal correlation with the majority of retinal ganglion cell death.

Neuregulins may also play an important role in the growth of retinal neuronal dendrites. Ganglion cells form axons prior to their migration from the ventricular surface; however, the first dendrites form only after the cell somas have completed their migration to the ganglion cell layer (Perry and Walker, 1980; Maslim et al., 1986). Thus, in early embryonic retinas, there are ganglion cells that are beginning to develop dendrites as well as ganglion cells that are still in the 'bipolar' phase. Maslim et al., (1986) have classified developing ganglion cells into three phases: I, axon growth and soma migration; II, active dendrite growth; and III, passive or interstitial growth. The expression of erbB2/neu correlates temporally with the second stage of retinal ganglion cell development, which in the rat, extends from embryonic day 17 to postnatal day 4/6. This stage is characterized by very active dendrite extension and branching. Thus, our data are consistent with a role for neuregulin in the regulation of the timing of initiation or the total number of dendrites elaborated by the retinal ganglion cells during development.

Might neuregulins be involved more generally in the regulation of synaptogenesis in the CNS? In muscles, neuregulin increases the expression of acetylcholine receptors and other

molecules involved in nerve/muscle synapses (Chu et al., 1995; Corfas et al., 1993; Corfas and Fischbach, 1993; Jo et al., 1995). Consistent with this hypothesis, a recent report has shown ARIA-like immunoreactivity in synaptic zones in the rat cerebellum (Sandrock et al., 1995). In the rat retina the timing of synaptogenesis in inner plexiform layer has been studied with electron microscopy. Conventional synapses – presumably between amacrine cells and ganglion cells – are first observed on postnatal day 11, while ribbon synapses – presumably from bipolar cells – develop on day 13 (Horsburgh and Sefton, 1987). Thus, neuregulin expression precedes the development of retinal synapses and erbB2/neu is downregulated prior to morphological evidence of synapses. However, ganglion cells in the rat retina are responsive to neurotransmitters (including glutamate, acetylcholine, and GABA) during the late embryonic and neonatal period (Rorig and Grantyn, 1993). Therefore, it is possible that neuregulin and erbB2/neu are important for the regulation of neurotransmitter receptor expression in retinal ganglion cells.

Our data support roles for neuregulin in neuronal survival and neurite growth. We have also examined the effects of neuregulin in cultures of mitotically active retinal progenitor cells and have not found any evidence for mitogenic effects of this factor on retinal progenitor cells (Reh and McCabe, unpublished observations). This is also consistent with the lack of erbB2, erbB3 and erbB4 immunoreactivity in the progenitor zone of the developing retina. Instead, it appears that only after the progenitor cells have become postmitotic neurons and migrated away from the progenitor zone, that they express any of the receptors for neuregulin and become responsive to the factor for later stages of differentiation.

Our results further raise the possibility that neuregulin may act in the nervous system in a paracrine or autocrine manner, similar to that proposed in other tissues (Marikovsky et al., 1995). Myotubes have been shown to both synthesize and respond to ARIA, consistent with autocrine regulation of acetylcholine receptor expression (Moscoso et al., 1995). Recent results from analysis of targeted mutations of the genes for neuregulin, erbB4 and erbB2/neu are also consistent with either local paracrine or autocrine actions of neuregulins (Meyer and Birchmeier, 1995; Gassman et al., 1995; Lee et al., 1995). Migrating crainial neural crest cells express both neuregulin and two of the putative receptors, erbB2/neu and erbB3, suggesting that local autocrine or paracrine signalling occurs among these cells during their development (Meyer and Birchmeier, 1995; Lee et al., 1995). Moreover, deletion of the genes for either neuregulin or erbB2/neu results in the loss of the neural crest derived cranial nerve ganglia (Meyer and Birchmeier, 1995; Lee et al., 1995). The expression of neuregulin in embryonic and neonatal rat retina is predominantly in the ganglion cell layer, where the putative receptors for neuregulin are also expressed during this period. While it is impossible to rule out either a paracrine or autocrine action, two observations from our cell culture experiments suggest that neuregulin acts in a paracrine manner. First, we found that rhGGF2 was necessary to support the growth of neurites from the majority of isolated ganglion cells in culture, suggesting that if neuregulin can act in an autocrine fashion, the amount of factor produced by the cells in vitro is limiting. Second, we found that ganglion cells that were not isolated, but instead grew adjacent to other ganglion cells, frequently had extensively branched neurites in the control wells, without exogenous GGF. Thus neuregulin may normally act on neighboring cells to stimulate their neurite growth. Alternatively, it is possible that other factors released by these neurons have similar effects.

Several other factors, including FGFs and neurotrophins, have been previously shown to promote survival of retinal ganglion cells both in vitro and in vivo (see Jelsma and Aguayo, 1994, for review), and in some cases these factors are active over the developmental period corresponding to the time of naturally occurring cell death. In addition, BDNF and NT-4/5 have been shown to promote the regeneration of neurites from adult rat retinal explants (Cohen et al., 1994). Therefore, some of the actions of neuregulins may overlap with the effects of other peptide growth factors. However, due the the restricted temporal expression of erbB2/neu and the correlation with survival and neurite outgrowth actions of neuregulin during this period, it is likely that these molecules are playing a key role in the control of neuronal development in the retina.

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