Activation of epidermal growth factor receptors directs astrocytes to organize in a network surrounding axons in the developing rat optic nerve

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Abstract

In postnatal developing optic nerves, astrocytes organize their processes in a cribriform network to group axons into bundles. In neonatal rat optic nerves in vivo, the active form of EGFR tyrosine kinase is abundantly present when the organization of astrocytes and axons is most actively occurring. Blocking activity of EGFR tyrosine kinase during the development of rat optic nerves in vivo inhibits astrocytes from extending fine processes to surround axons. In vitro, postnatal optic nerve astrocytes, stimulated by EGF, organize into cribriform structures which look remarkably like the in vivo structure of astrocytes in the optic nerve. In addition, when astrocytes are co-cultured with neonatal rat retinal explants in the presence of EGF, astrocytes that are adjacent to the retinal explants, re-organize to an astrocyte-free zone into which neurites grow out from the retinal tissue. We hypothesize that in the developing optic nerve, EGFR activity directs the formation of a histoarchitectural structure of astrocytes which surrounds axons and provides a permissive environment for axon development.

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Introduction

In the developing central nervous system, the epidermal growth factor receptor (EGFR) plays a critical role. EGFR is linked to the mitotic responsiveness and the cell fate choice of neuroprogenitors (Burrows et al., 1997; Lilien and Raphael, 2000; Lilien and Wancio, 1998), neurogenesis (Blum, 1998), and astrogliogenesis (Rabchevsky et al., 1998). During late embryonic development, EGFR acts as an astrocyte-inducing signal to promote astrocyte development (Burrows et al., 1997), perhaps by regulating the responses of progenitors to extrinsic signals, such as leukemia inhibitory factor (Viti et al., 2003). Specific populations of cells, for example in the telencephalon, migrate in response to EGFR ligands by a chemoattractive mechanism (Caric et al., 2001). EGFR ligands, such as TGFα and EGF, provide neurotrophic effects to increase neurite extensions (Wildering et al., 2001).

Depending on the genetic background (Kornblum et al., 1998; Miettinen et al., 1995, 1999; Sibilia and Wagner, 1995; Threadgill et al., 1995), Egfr knockout are embryonically lethal, die in the neonatal period due to severe immaturity of several epithelial organs, or survive for a time postnatally but develop severe degenerations of the olfactory bulb, neocortex, and cerebellum. The neurodegeneration in the Egfr−/− mice is characterized by massive cell apoptosis involving both neurons and astrocytes (Kornblum et al., 1998; Sibilia et al., 1998). In developing Egfr−/− mice, astrocytes do not migrate into the cortex (Kornblum et al., 1998), consistent with the suggestion that EGFR activation signals developmental functions in astrocytes.

Our previous work found that activation of EGFR dramatically changes the morphological characteristics and gene expression of cultured astrocytes (Liu and Neufeld, 2004). EGF-treated adult rat and human optic nerve astrocytes in vitro form cribriform structures, which are similar to the histological structure of astrocytes in optic nerve heads in vivo. Based on these findings, we hypothesized that the...
EGFR signal pathway may regulate the morphogenesis of astrocytes in optic nerves during development.

As ocular development proceeds, optic cup and optic stalk develop into retina and optic nerve, respectively (Kuwabara, 1975). The first axons of the retinal ganglion cells exit the eye into the optic stalk through an opening at its base where the choroid fissure develops. Neuroepithelial cells of the optic stalk give rise to the first optic nerve glial cells and to cells lining the choroid fissure. The retina on either side of the choroid fissure enlarges and fuses to form the optic disk. The neuroepithelial cells around the optic disk extend into the optic nerve head and guide axons into the optic nerve (Oster and Sretavan, 2003; Stuermer and Bastmeyer, 2000). The determination and functions of these neuroepithelial cells are influenced by Pax-2, Shh, netrin-1, and members of the Slit family (Dakubo et al., 2003; Lemke, 2001; Macdonald and Wilson, 1996; Macdonald et al., 1995). The neuroepithelial cells, driven by bFGF, divide and differentiate into GFAP-positive astrocytes in the optic nerve by E19 (Mi and Barres, 1999).

Retinal ganglion cells first project axons through glial channels on the retinal surface before reaching the optic stalk on E14 (Sefton et al., 1985). The axonal connections of the retinal ganglion cells are made to the brain and are dependent on axon guidance molecules in the later stages of optic nerve development in most mammals (Dallimore et al., 2002). At birth, the optic nerve contains approximately twice the number of axons of the adult animal. During the first week of birth, astrocytes extend processes to separate axon bundles and over 50% of the axons degenerate (Crespo et al., 1985). Myelination of the axons posterior to the optic nerve head begins on day P5 and continues until the end of the second postnatal week (Kuwabara, 1975). About the same time, an astrocyte lattice develops in the retina mediated by platelet-derived growth factor (Fruttiger et al., 1996). By the end of the 3rd postnatal week, the rat optic nerve is developed into its adult form.

In the present studies, we have found that EGFR is highly expressed and activated in optic nerve head astrocytes in the late stage of optic nerve development in rats. Blocking EGFR function postnatally causes significant disorganization of astrocytes and axons in the optic nerve. Our findings support the hypothesis that the EGFR signal pathway directs the formation of a histoarchitectural structure of astrocytes that is needed for the proper maintenance and organization of axons as the optic nerve nears the end of its developmental period.

Materials and methods

Immunohistochemistry of perinatal optic nerve development

Developing pups of Wistar rats were sacrificed at embryonic day 19 and at postnatal days: 1, 3, 6, 9, 14, 17, or 21. Six eyes from three rats at each time point were processed for histology or immunohistochemistry. The eyes were enucleated and fixed in 4% paraformaldehyde. The optic nerves were dissected free of surrounding tissues. Fixed tissue was washed in 0.2% glycine in phosphate-buffered saline (PBS), pH 7.4, dehydrated, embedded in paraffin, and oriented for 6 μm sagittal or cross-sections. Cross-sections of the optic nerves were cut anterior (the area proximate to the eye sclera layer). Three sections per eye were randomly chosen for standard H&E staining for observation of histological structure. The neighboring slides of each of the three sections were used for immunohistochemical studies.

The paraffin tissue sections were deparaffined, rehydrated, and washed completely with 0.1 M glycine–Tris solution. To block nonspecific binding, the tissue sections were pre-incubated with 20% donkey serum at room temperature for 30 min. Immunohistochemistry was performed using specific primary antibodies against p-EGFR (Santa Cruz Biotechnology Inc, Santa Cruz, CA; goat polyclonal, working dilution 1:200), glial fibrillary acidic protein (GFAP) (Chemicon International, Temecula, CA, mouse monoclonal, working dilution 1:200), EGF (goat polyclonal, working dilution 1:100) and TGFα (rabbit polyclonal, working dilution 1:25), tau (goat polyclonal, working dilution 1:150) (Santa Cruz Biotechnology Inc) or neurofilament (SMI-31, Sternberger Monoclonals Inc, Lutherville, MD; working dilution 1:800) and the Vectastain Elite ABC kit (Vector Labs, Burlingame, CA), using diaminobenzidine as substrate. Hematoxylin was the counter stain. Negative controls were performed in parallel by pre-incubation of the primary antibody at 4°C overnight with its specific blocking peptide.

Immunoblots

Rat optic nerve tissues were dissected from the eye globe to the optic chiasma and obtained from two to three pups of one litter at postnatal days: 1, 3, 6, 9, 14, and 21. Three different litters of animals were used. Rat optic nerve tissues were lysed in buffer (20 mM HEPES, pH 7.0, 10 mM KCl, 2 mM MgCl2, 0.5% Nonidet P40, 1 mM Na3VO4, 1 mM PMSF, 0.15 U ml⁻¹ aprotinin) and homogenized. Protein concentration was determined using the Bradford colorimetric assay. Twenty micrograms of each protein lysate were loaded in each lane in sample buffer (2% SDS, 10% glycerol, 0.001% bromophenol blue, 1% DTT, and 0.05 M Tris–HCl, pH 6.8), separated on 10% SDS-PAGE, and transferred to a nitrocellulose filter. The blots were blocked with 5% nonfat milk in PBS and then incubated with specific rabbit polyclonal antibody against EGFR (Santa Cruz Biotechnology Inc; working dilution 1:200), p-EGFR (Santa Cruz Biotechnology Inc, goat polyclonal, working dilution 1:100), GFAP (Sigma-Aldrich Corp, St Louis, MO; rabbit polyclonal, working dilution 1:200), neurofilament (SMI-31, Sternberger Monoclonals Inc; mouse monoclonal, working dilution 1:2000) or actin (Sigma-Aldrich Corp., mouse monoclonal, working dilution 1:5000) followed by
peroxidase-conjugated goat anti-rabbit IgG2a and the enhanced chemiluminescence detection system (Amersham Life Science Inc, Arlington Heights, IL).

**Administration of AG1478 in vivo**

After birth, all pups were weighed, divided into four groups and marked for identification. One group (n = 12) was treated daily with AG1478, a very potent and selective inhibitor of EGFR (Ellis et al., 2001; Liu et al., 1999), at a dose of 50 mg/kg (Rice et al., 1999) in DMSO vehicle by subcutaneous injection, a group of pups was treated similarly at a dose of 5 mg/kg AG1478, a group (n = 12) was treated similarly with the DMSO vehicle, and a group (n = 12) was untreated. The animals were weighed daily and closely followed. Four different litters of rats were used. At 10 or 21 days postnatal, six animals of each group were sacrificed. For each rat, one optic nerve was processed as paraffin cross-sections for histology using H&E or for immunohistochemistry using antibodies against pEGFR, GFAP, or neurofilament (SMI-31) as described above, and the other optic nerve was processed for electron microscopic examination.

**Electron microscopic examination**

Optic nerves including the posterior eyes were carefully dissected and fixed in 2% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M phosphate buffer. The tissues were postfixed in 1% osmium tetroxide for 1 h at 4°C. After further trimming and orientation under a dissecting microscope, the small pieces were dehydrated in a graded series of ethyl alcohol (50–100%), embedded in an Epon 812 and Araldite 6005 mixture and oriented for cross-sections of optic nerves. Thick (0.5–1.0 μm) sections were stained with alkaline toluidine blue for light microscopic observation to locate the section-cutting level of the optic nerve at the area proximate to the scleral layer. Ultrathin (70–80 nm) sections were placed on a 2 × 1 nm cooper grids and stained with uranyl acetate and lead citrate. Sections were examined using a transmission electron microscope (Hitachi H-7500).

**Cell cultures**

Rat optic nerve astrocytes were derived from the anterior portions of the rat optic nerve from six Wistar rats on postnatal day 10 (Charles River, Chicago, IL) by the same procedure as for human optic nerve astrocytes as described previously (Hernandez et al., 1988). The posterior pole of the eye was dissected and the optic nerve head was freed from sclera and other neighboring tissues. The optic nerve head was sliced sagittally, and under a dissecting microscope, the anterior portion of the optic nerve was carefully dissected from the pre- and post-laminar regions. From each eye, two or three explants were obtained. The explants were put into T 25 cm² plastic tissue culture flasks, which had been conditioned with DMEM/F-12 supplemented with 10% fetal bovine serum. The first passage cells were characterized by immunostaining of GFAP and neural cell adhesion molecule and other cellular markers (HLA-DR, α-smooth muscle actin and von Willebrand factor) to distinguish them from other cell types from the optic nerve. The primary cell cultures were purified for astrocytes by growing the cells for 1 week in modified astrocyte-defined, serum-free medium (ADM, Clonetics) containing forskolin, which suppresses the growth of fibroblasts. The second-passage cell cultures, which had over 95% cells positive for GFAP, were grown to 60–80% confluency and serum-starved in ADM for 1 week before being used for the following experiments.

EGF (final concentration 10 or 100 ng/ml) (Kubota et al., 2001) (Sigma-Aldrich Corp.) was added to the cell media for 12, 24, 48, 72 h, or 6 days.

**Astrocyte/retinal explant co-cultures**

Rat optic nerve astrocytes were grown on coverslips to confluence and serum-starved for 3–7 days. Retinal explants were obtained from neonatal (P2) Wistar rats. After rats were anesthetized, the eyes were removed and the retinas were dissected from surrounding tissues in cold DMEM/F-12 cell media. The isolated retinas were carefully cut into approximately 1 mm² pieces by sharp blades. The retinal explants were attached to the astrocyte monolayer with the retinal ganglion cell layer facing the astrocytes. Appropriate amounts of cell growth media DMEM/F-12 without serum were added to immerse the astrocytes and the retinal explants but to not float the explants. Retinal explants were also placed on poly-l-lysine coated coverslips as controls. When present, EGF (100 ng/ml) was added to the cell growth media when retinal explants were placed into the culture media. The cultures were maintained at 37°C in 5% CO₂/air in an incubator for 3 days.

**Immunocytochemistry**

The co-cultures were fixed in 4% paraformaldehyde and processed for immunocytochemistry for neurofilaments (SMI-31, working dilution 1:200), tau (Santa Cruz Biotechnology Inc; goat polyclonal, working dilution 1:50), GFAP (rabbit polyclonal, working dilution 1:50) or phalloidin (Molecular Probes, Eugene, OR; Rhodamine red-X conjugated, working dilution 1:50). Double-staining for tau and phalloidin was used to show the histoarchitecture of the co-cultures. As described previously (Liu and Neufeld, 2000), cells grown on coverslips were fixed in 4% paraformaldehyde at 4°C for 30 min, washed in PBS and treated with 0.5% FBS/0.2% Triton X-100/0.5% glycine in PBS for 20 min. The coverslips were incubated with specific primary antibodies. After washing several times with PBS, the coverslips were incubated with appropriate secondary antibody. For double immunofluorescent staining, the coverslips were sequentially incubated with the second primary...
antibody and the second appropriate secondary antibody. After washing several times with PBS, the coverslips were mounted in ProLong (Molecular Probes Inc.). Secondary antibodies: goat anti-mouse Oregon green (1:400), goat anti-rabbit Rhodamine red-X conjugated IgG (1:1000), horse anti-goat FITC-X conjugated IgG (1:50) were purchased from Molecular Probes.

Slides were observed and photographed using a microscope (Olympus AX70, Tokyo, Japan) equipped with a digital camera (Spot, Diagnostic Instruments Inc., Sterling Heights, MI).

**Neurite growth zone analysis**

After hematoxylin–eosin (H&E) staining, the retinal explants were photographed using a 1.25× lens on a microscope equipped with a digital camera. Using Optimas 6.2 image analysis software, the micrographs of the retinal explants were measured and quantitated for the area of the zone covered by the growing neurites. The area of the neurite growth zone was normalized to the perimeter of the retinal explant. Twelve explants for each experimental condition and control group from each of three independent experiments were measured. All data were statistically analyzed using Student’s t test.

**Results**

**Activated EGFR in the developing rat optic nerve head**

To obtain a temporal sequence of the activation of EGFR and the histological structure of the developing optic nerve head, a series of embryonic and postnatal (from day E19 to P21) rat optic nerve head sections were studied for activated EGFR (p-EGFR), EGFR ligands (EGF and TGFα), structure of astrocytes (GFAP), and maturity of axons (neurofilament SMI-31).

In optic nerve heads of day E19, cells were dispersed randomly throughout the tissue. Labeling for GFAP was weak, indicating that the glial precursor cells had not fully matured to be GFAP-positive astrocytes (Fig. 1A). Labeling for p-EGFR showed that the cells in the optic nerve head had minimal levels of the active form of EGFR (Fig. 1B). EGF was not detectable (Fig. 1C); however, labeling for another EGFR ligand, TGFα, was weakly positive in the cell bodies (data not shown). Labeling for neurofilaments was negative in optic nerves of day E19 (Fig. 1D).

Observations on sequential tissue sections of optic nerve heads from day E19, P1, P3, and P6 demonstrated that the level of p-EGFR and EGF gradually increased and GFAP-positive labeling became more intense. In optic nerve heads of day P6, cells had increased in number and were intensely labeled for GFAP. The GFAP-positive astrocytes formed a cribriform network with a fine lattice of astrocyte processes in the optic nerves on day P6 (Fig. 1E). At the same time, abundant labeling for p-EGFR was present (Fig. 1F) and EGFR ligands, EGF (Fig. 1G) and TGFα (data not shown) were also abundantly detected in the cell bodies. However, the labeling for neurofilament was negative (Fig. 1H). The labeling for GFAP, p-EGFR, and EGFR ligands gradually decreased in intensity in the optic nerves through days P9 to P21. At day P9 when a full complement of axons is known to be present in the optic nerve, neurofilament SMI-31 was minimally detectable. Thereafter, neurofilament SMI-31 increased in amount, filling the spaces between the cribriform network of astrocytes.

On day P21, the optic nerves were densely packed with cells but the labeling for GFAP in the astrocytes was moderate (Fig. 1I) and the labeling for p-EGFR was faint (Fig. 1J). The labeling for EGF was weak and mainly in the cells near capillaries (Fig. 1K). On day P21, labeling for neurofilament SMI-31 was very intense and appeared as bundles in the cross-sections of optic nerve heads (Fig. 1L). These neurofilaments, which were detected in axons at the later stage of optic nerve development, apparently demonstrate mature axons.

These immunohistochemical results were confirmed by immunoblot (Fig. 2). The intensity of the immunoblot bands for GFAP gradually increased after birth, peaking on P6-P9 and then decreased to a moderate level after P14. By immunoblot, p-EGFR was detected on P1, highly increased in amount on P3 and P6, and became undetectable after P9. Although both the immunohistochemical and immunoblot studies for p-EGFR indicated that EGFR was highly phosphorylated around P6, an intense immunoblot band for EGFR was detected in the optic nerves on P1 and the protein levels of EGFR gradually decreased through P3 to P9, becoming barely detectable after P14. Neurofilaments were not detectable on P1, but were clearly detected on P9 and became abundant on P21. Our studies using immunoblot and immunohistochemistry show that the activation of the EGFR was concurrent with the formation of the fine cribriform network of astrocytes in the postnatal developing rat optic nerves. The appearance of neurofilament SMI-31, as a marker for the maturity of the cytoskeleton of axons, was sequentially later than the peak time of the activation of EGFR and the organization of the cribriform structure of astrocytes in the developing optic nerve.

**Activation of EGFR causes organization of postnatal astrocytes into cribriform structures in vitro resembling the tissue structure of astrocytes in optic nerves in vivo**

Previously, we demonstrated that adult astrocytes from rat optic nerve heads elongate and form cribriform structures with cell-free spaces in response to activation of EGFR in vitro (Liu and Neufeld, 2004). To confirm these observations in astrocytes from developing optic nerves, we cultured astrocytes from optic nerves of day P10 rats. In the absence of exogenous EGF, rat optic
nerve astrocytes, grown in serum-free medium, were flat, polygonal and formed a monolayer. Very few astrocytes were elongated (Fig. 3A).

The morphology of optic nerve P10 astrocytes changed significantly after addition of EGF to the media. Within 12 h, the cells became elongated with very long, thin processes that crossed over several neighboring cells. A few astrocytes remained flat and polygonal in shape. In addition to the shape changes, the astrocytes gradually lost the monolayer arrangement to form a cribriform architecture. Within 12–24 h of the initial exposure to EGF, the astrocytes appeared to stretch out further in the direction of their long processes. Gradually, the elongated astrocytes piled up in local areas, developed interlaced processes and circumferentially surrounded cell-free spaces. After 3 days of treatment with EGF, the astrocytes throughout the culture dish clearly reorganized to produce a cribriform structure that had multilayers of elongated astrocytes interweaving their processes to form a network surrounding large cell-free spaces (Fig. 3B).

Comparing the in vitro architecture of EGF-treated astrocytes (Fig. 3B) to the in vivo histological organization of astrocytes (Fig. 1E), there were striking similarities in morphology between the cribriform structure formed by EGF-treated, P10 astrocytes in vitro and the postnatal organization of astrocytes in optic nerve heads in vivo.

Inhibition of EGFR activity caused defects in the postnatal development of both astrocytes and axons

To demonstrate the function of EGFR in developing optic nerves, we performed pharmacological experiments to determine whether inhibition of activation of EGFR would alter development. We used AG1478, a specific inhibitor of EGFR tyrosine kinase, which has been used...
previously in vivo to block EGFR (Rice et al., 1999). Pups treated with AG1478 at 5 mg/kg for the 3-week postnatal period appeared to gain weight and to develop normally. Pups treated with AG1478 at 50 mg/kg gained less weight than controls and were not examined further.

Electron microscopic examinations of cross-sections of optic nerve heads of AG1478-treated rats revealed significant deficiencies of the cellular network of astrocytes and abnormalities of axons when compared with normal rats. At P1, before treatment with AG1478, optic nerves had already fully filled with small diameter axons, which were separated into large bundles by thick processes of astrocytes (data not shown). In normal, developing optic nerve heads at P10, astrocytes had extended branches into axons to group the axons into bundles (Fig. 4A); however, within each axon bundle, many axons were tightly packed together without separation by fine processes of astrocytes (Fig. 4B). On the cross-sections of normal optic nerves at P10, the diameters of axons varied and appeared increased in size compared with that on P1. The astrocytes contained numerous filaments, microtubules, and various microorganelles.

In AG1478-treated rats at P10, there were abundant vacuolated spaces in axon bundles (Fig. 4C). Vacuolation of axons appeared as large empty spaces without internal structures inside the nerve bundles (Fig. 4D) and also was observed inside individual axons (Fig. 4E), which indicated abnormal, perhaps degenerating axons. The vacuolated spaces in axon bundles were observed in the optic nerves of all AG1478-treated rats on P10 by electron microscopy. In addition, there were fewer processes of astrocytes in the optic nerve heads of AG1478-treated rats and the astrocytic processes were irregular compared to normal optic nerve heads at P10.

In normal optic nerve heads at P21, the fine network of astrocytes was formed. Astrocytes extended long, thin processes to further group axons into bundles (Fig. 4F). Inside the axon bundles, astrocytes had extended branches of very fine processes to surround the axons (Fig. 4G). By the shape of axons in the cross-section, axons appeared regularly aligned with straight paths. However, in the optic nerve heads of AG1478-treated rats at P21, the astrocyte network and the axons were disorganized. The processes of astrocytes were short, thick, and lacked filaments. Axons were not regularly grouped into bundles by fine processes of astrocytes (Fig. 4H). Furthermore, the axons had irregular shapes on cross-sections of the optic nerves (comparing Figs. 4H and F). We did not observe vacuolated spaces in nerve bundles in the AG1478-treated optic nerve heads at P21 as were present at P10.

By light microscopy, defects of astrocytes and axons are also demonstrated in the optic nerve heads of AG1478-treated rats. On postnatal day 1, when rat pups were started on the treatment with AG1478, astrocytes have formed an initial sparse network and axons were grouped into very large bundles in the optic nerves. Nevertheless, postnatal treatment, starting on P1, with AG1478 significantly affected astrocytes to further develop the fine lacing network and to group axons into small bundles in the optic nerve head. Comparing the histological appearances with H&E staining (Figs. 5A and E, B and G), the fine, lacy processes of astrocytes are clearly absent in the optic nerves of the drug-treated animals at P10. Also, in the optic nerves of AG1478-treated rats at P10, there were
fewer astrocytes. In normal optic nerve heads at P10, labeling for GFAP was moderate and demonstrated that the astrocytes had fine interlacing cell processes (Fig. 5C).

In AG1478-treated animals, the astrocytes were intensely positive for GFAP in the cell bodies, but there was no formation of the fine network of astrocytic processes.
Labeling for neurofilament showed axons filling in the spaces provided by the network of fine astrocytic processes in the normal developing optic nerve heads (Fig. 5E). However, labeling for neurofilament was negative on P10 in the optic nerve heads from AG1478-treated rats (Fig. 5J). Labeling for p-EGFR confirmed efficacy of treatment with AG1478 by a much lower level of activation of EGFR in the AG1478-treated animals (compare Figs. 5D and I).

These data suggest that activation of the EGFR pathway is critical for optic nerve head astrocytes to develop and to organize a fine network of processes and is needed for the proper maintenance and maturation of the axons during the postnatal development of the optic nerve.

**EGF stimulated astrocytes re-organize into cell-free spaces and promote neurite outgrowth**

We tested how astrocytes respond to EGF stimulation in the presence of growing neurites of retinal explants. We placed neonatal retinal explants on monolayers of optic nerve astrocytes. The neurites that grow from such retinal explants are predominantly axons of the retinal ganglion cells. To identify the cells in the co-cultures, we used double-labeling immunocytochemistry for neurofilament SMI-31 or tau to demonstrate neurites and GFAP or phalloidin to localize astrocytes.

In co-cultures which were not treated with EGF (Fig. 6A), although there were astrocytes present initially, and presumably, glia growing from the retinal explant, no neurites extended from the retinal explant cultured in basic serum-free media in 3 days. In control cultures in which retinal explants were placed on poly-l-lysine-coated glass in the absence of astrocytes, no neurite outgrowth occurred in the absence of EGF (Fig. 6B) and only modest neurite outgrowth occurred in the presence of EGF, but the absence of astrocytes (Fig. 6C).

In the co-cultures of astrocytes and neonatal retinal explants that were exposed to EGF, astrocytes which were adjacent to the explants re-organized into a cell-free zone that circled the explants. In addition, there was rapid and prodigious growth of neurites from the retinal explants into the cell-free zone ringed by astrocytes (Fig. 6D). The cell-free zone containing neurites was formed circumferentially around the explant, between the original retinal explant and the organized astrocytes, and extended wider during the 72 h of EGF treatment. At 72 h of exposure to EGF, the extended neurites from the retinal explants were curved along the edge of the cell-free zone.
and did not extend over the astrocytes. These observations suggested that under the regulation of the EGFR pathway, there were attractive signals to promote neurite outgrowth toward astrocytes and also repulsive signals to limit the outgrowing neurites to the spaces surrounded by astrocytes.

In EGF-treated co-cultures of astrocytes and neonatal retinal explants, the growth of neurites was so great that we used a surrogate endpoint for quantitation rather than trying to trace each neurite. To compare the outgrowth of neurites of experimental and control retinal explants, we quantitated the “neurite growth zone” as indicated in Fig. 6E. In EGF-treated co-cultures, the neurite growth zone was significantly greater than the control groups (P < 0.05) (Fig. 6F). These results, which greatly underestimate the prodigious neurite outgrowth in response to astrocytes treated with EGF, nevertheless support the hypothesis that the activation of EGFR directs astrocytes to surround axons and to produce a permissive environment for axonal growth.

Fig. 6. Effect of EGF on the growth of neurites in co-cultures of rat, neonatal retinal explants. Retinal explants with (A) or without (B) astrocytes did not extend neurites within 72 h of culture. In the presence of EGF but the absence of astrocytes (C), EGF had a modest effect on the extension of growing neurites. In the presence of EGF and astrocytes (D), retinal explants extended dense networks of growing neurites in 72 h. EGF induced astrocytes to organize an astrocyte-free zone into which the neurites extended. The neurite growth zone surrounding the entire retinal explant was measured (E), by the following equation: (outer area of neurite extension) – (area of retina explant) / perimeter of retina explant. Data were calculated from digital photomicrographs by Optimas software. The neurite growth zone was significantly greater in co-cultures treated with EGF (F, mean ± SEM of three experiments). Immunolabeling: green, tau; red, phalloidin. Magnification: A–D, ×200; E, ×100.

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Discussion

The functional significance of our observations may relate to a previously unsuspected role for EGFR in the developing optic nerve. Our results show that late in the developing optic nerve, the activated EGFR pathway directs the organization of astrocytes that ultimately affects the formation of axon bundles. The EGFR signal pathway may have a similar role in the development of other white matter regions in the central nervous system.

In the normal development of the rat optic nerve, activation of EGFR coincides with dramatic morphological changes in astrocytes. EGFR is present on E19, peaks in the first postnatal week and remains detectable until the 3rd postnatal week. During this period, activation of EGFR occurs and astrocytes complete the histarchitectural structure of the fine cribiform network that groups axons into bundles, and the axons mature. There are significant defects of the optic nerve when EGFR is inhibited postnatally by a specific EGFR tyrosine kinase inhibitor, AG1478. In the optic nerve heads of rats treated with AG1478 postnatally, astrocytes do not form the fine cribiform network grouping axons into small bundles and when observed on postnatal day 10, there are abundant vacuolation spaces in axon bundles and the axons contain less neurofilaments. Our results suggest that activation of EGFR is critical for the formation of the cribiform network of astrocytic processes surrounding axons and for the maintenance, maturation, and organization of axons in the later development of the rat optic nerve.

As shown by immunolabeling for p-EGFR, activation of EGFR is significantly blocked in the optic nerves of rats treated with AG1478. Our pharmacological approach administered AG1478 to rat pups and may not have completely inhibited EGFR tyrosine kinase activity in rat optic nerve heads during the postnatal development. Loss of function of EGFR has been studied by Egfr gene knockouts. However, the function of EGFR in the later development of optic nerves cannot be studied using knock out mice because the Egfr−/− animals are embryonically lethal or have severe central nervous system malformation due to the critical functions of EGFR in early embryonic development.

Our in vitro experiments support our hypothesis that postnatal activation of EGFR causes astrocytes to form a cribiform network and organize the axons. In vitro, activation of EGFR induces rat optic nerve astrocytes to form cribiform structures, which are remarkably similar to the in vivo tissue structure of astrocytes in optic nerve heads. In astrocyte/retinal explant co-culture, EGF stimulates the astrocytes to re-organize and to form cell-free spaces created adjacent to the retinal explants to provide the path and space for the outgrowing neurites. In addition, there was significant promotion of neurite outgrowth by EGF when astrocytes were present. The interaction between astrocytes and outgrowing neurites in response to activation of the EGFR pathway may involve both attractive and repulsive signals released by astrocytes that affect the growth cones of neurites.

Our in vitro results were obtained using EGF as the EGFR ligand. Ligands that bind to EGFR include EGF, TGFα, epiregulin, β-cellulin, and amphiregulin. We do find EGF and TGFα in rat perinatal optic nerve tissues by immunohistochemistry. TGFα knockout mice have abnormal eyes (Luetteke et al., 1993) suggesting that the expression of EGFR ligands affects the development of the eye. In addition, many axon guidance molecules, such as netrin (Yu and Bargmann, 2001), Slit (Rothenberg et al., 1990), laminin (Engel, 1992), and tenascin-C (Saga et al., 1991), contain EGF repeats in their protein structures. The EGF-like repeats of human tenascin-C can function as EGFR ligands which stimulate EGFR autophosphorylation and induce mitogenesis in an EGFR-dependent manner (Swindle et al., 2001). EGFR on astrocytes may function as a receptor for these guidance molecules and thus participate in the guidance of axon growth and fasciculation.

Finally, there are striking similarities between our observations on the mammalian eye and the developmental regulation by the EGFR pathway in Drosophila eyes and peripheral nervous system. In Drosophila melanogaster, the retina is sculpted into a pattern of lattice cells surrounding the photoreceptors in the ommatidia of the eye. Activation of Drosophila EGFR (dEGFR) by its ligand, Spitz, is a life signal to lattice cells causing excessive proliferation that is apparently overcome by a death signal from Notch causing apoptosis. This mechanism sets the photoreceptor in the precise hexagonal pattern of lattice cells of the adult eye (Brachmann and Cagan, 2003). In Drosophila peripheral nervous system development, the EGFR pathway in glial cells guides sensory axon pathfinding and is required for the normal organization of sensory neurons with peripheral glia (Sepp and Auld, 2003). The use of the EGFR pathway to organize certain regions of the mammalian neural system by non-neural cells may have been conserved throughout phylogeny.

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References


