Structural changes in the developing retina maintained *in vitro.*

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Abstract

The present study examined the emergence of structural remodeling in explanted neonatal rat retina. Immunohistochemical analysis demonstrated signs of glial and neuronal remodeling after 11 days *in vitro* and included the activation of Müller cells, the formation of ectopic neuropil areas and sprouting of photoreceptor terminals. We also observed that cholinergic and GABA-ergic amacrine cells displayed signs of disorganized laminations. These results demonstrate that retinal culturing initiates structural changes that show morphological similarities to glial and neuronal remodeling identified in retinitis pigmentosa retinas and experimentally detached retinas.

Keywords: Retina, In vitro, photoreceptor sprouting, remodeling, Müller cells.

1. Introduction

There are considerable data to show that explants of embryonic and neonatal rat retina differentiate into a laminated organization similar to the one found in age-matched normal retina (LaVail, and Hild, 1971; Sparrow, Hicks, and Barnstable, 1990). Several immunohistochemical analyses indicate a proper differentiation of different retinal cell classes and their termination patterns in the inner retina of cultured retinas (Johansson, Bruun, Grasbon, and Ehinger, 2000; Pinzón-Duarte, Kohler, Arango-Gonzáles, and Guenther, 2000). These studies were focused on developmental events in retinas kept in culture for at least 10 days. Massive axotomy-induced apoptosis of ganglion cells occur within days after explantation (Rehen, Varella, Freitas, Moraes, and Linden, 1996; Mertsch, Hanisch, Kettenman, and Schnitzer, 2001; Manabe, Kashii, Honda, Yamamoto, Katsuki, and Akaike, 2002; Engelsberg, Ehinger,Wassélius, and Johansson, 2004). Furthermore, retinal culture also results in an impaired development of photoreceptor outer segments (Pinzón-Duarte et al., 2000), reduced expression of cone opsin mRNAs (Liljekvist-Larsson, Törngren, Abrahamson, and Johansson, 2003) and a caspase-3 related degeneration of subsets of photoreceptor cells (Engelsberg et al., 2004). This raises the possibility that ganglion cell death and the varying degree of sensory deafferentiation may influence the developmental process *in vitro* and cause neuronal remodeling of the inner retina.

Neuronal remodeling *in vivo* occurs following ganglion cell death in rodents (Günhan-Agar, Kahn, and Chalupa, 2000; Reese, Raven, Gianotti, and Johnson, 2001) as well as photoreceptor degenerations in humans and rodents (Fariss, Li, and Milam, 2000; Jones, Watt, Frederick, Baehr, Chen, Levine, Milam, LaVail, and Marc, 2003; Marc, Jones, Watt, and Strettoi, 2003; Fletcher and Kalloniatis 1997). Experimentally induced photoreceptor degeneration by retinal detachment also triggers remodeling in

the Müller cell and surviving photoreceptor cell populations as well as in bipolar and horizontal cells (Lewis, Linberg, and Fisher, 1998, Fisher, and Lewis, 2003; Sakai, Calderone, Lewis, Linberg, Fisher, and Jacobs, 2003). Morphological studies of retinitis pimentosa (RP) retinas have revealed that structural changes in retinal architecture follow the sensory deafferentation and include sprouting of photoreceptor terminals, Müller cell hypertrophy, formation of ectopic neuropil areas, rewiring of neural circuits and migration of neuronal and glial cells (Li, Kljavin, and Milam, 1995; Jones et al., 2003; Marc et al., 2003). This type of remodeling follows a general pattern of events that proceeds through three phases (Jones et al., 2003; Marc et al., 2003). One experimental approach to study retinal remodeling of Müller cells, neuronal subsets and photoreceptor terminals as well as their expression of specific proteins is retinal detachment (Lewis, Linberg, and Fisher, 1998; Rex, Fariss, Lewis, Linberg, Sokal, and Fisher, 2002; Fisher and Lewis, 2003).

The aim of the present immunohistochemical analysis was performed to explore whether signs of anatomic changes arise in cultured retinas. We focused on structural changes that may be related to the insults caused by the experimental manipulation such as ganglion cell apoptosis and photoreceptor degeneration. Comparisons with recent studies of neuronal remodeling in cultured photoreceptor sheets, as well as RP and detached retinas are made. Although a substantial neuronal survival and development capacity has been demonstrated in retinal cultures, more subtle changes including neuronal sprouting and hypertrophic Müller cell processes that may form vitreal glial seals have not been evaluated.

2. Materials and methods

2.1 Animals

Postnatal pigmented rats (ScanBur, Sollentuna, Sweden) were used throughout this study. Data was collected from 4 different cultures and each culture consisted of 3-5 retinas and 1-2 littermate controls. Neonatals were sacrificed by decapitation, whereas P14 littermates were euthanized by CO_2 asphyxiation prior to decapitation. Experiments and animal care were performed according the ARVO convention for ophthalmologic animal experimentation and approved by the Swedish Government Committee for Animal Experimentation Ethics.

2.2 Tissue culture

Freshly enucleated eyes of postnatal day 3 (P3) rats were immersed in cold CO_2-independent medium (Gibco, Paisley, UK) and external tissue was removed. To prepare retinas with adherent retinal pigment epithelium (RPE), intact eyes were incubated in CO_2-independent medium containing 0.5 mg/ml proteinase K (Sigma, St Louis, MO, USA) at 37°C for 10-12 min. The neural retina with attached RPE was explanted onto a Millicell®-PCF 3.0 μm filter insert (Millipore, Bedford, MA, USA) with the vitreal side up. The explants were cultured in DMEM/F12 (Gibco) supplemented with 10% fetal calf serum, 1% penicillin/streptomycin, 2mM L-glutamine (Sigma) and maintained at 37°C with 95 % humidity and 5% CO_2. The specimens were kept in culture for 11 days to a stage that corresponded to postnatal day 14. Culture medium was changed every second day.

2.3 Tissue handling and antibodies

All retinal tissues were immersed in fixative consisting of 4 % paraformaldehyde in 0.1 M phosphate buffer (PB; pH 7.2) for 4 h at 4°C. Washed retinas were transferred from 10 to 25 % sucrose in 0.1 M PB, embedded for cryo-sectioning and cut at 10-12 µm. For immunohistochemical staining of cone bipolar cells, a rabbit polyclonal recoverin antibody was used at 1:10000 (generously provided by Dr. A. Dizhoor, Wayne State University, Detroit, USA). A monoclonal antibody against HuC/D (Molecular Probes, Eugene, OR) diluted 1:500 was used for a general staining of cells in the inner retina (see Ekström and Johansson, 2003). Cholinergic amacrine cells were labeled using either a goat polyclonal vesicular acetylcholine transporter protein antiserum (VAChT; Chemicon, Temecula, CA, USA) diluted 1:1000 or a rabbit polyclonal choline acetyl tranferase antiserum (ChAT; BioMetra, Göttingen, Germany) at 1:1000 dilution. GABA-ergic amacrine cells were labeled using a mouse monoclonal GABA antibody (Sigma) diluted 1:1000. A mouse vimentin monoclonal antibody (DAKO, Glostrup, Denmark) diluted 1:500 and a rabbit polyclonal glial fibrillary acidic protein (GFAP) antiserum (DAKO, Denmark) diluted 1:1000 were used to identify Müller cells. Photoreceptor cells and their terminals were identified using a mouse monoclonal antibody against rhodopsin (generously provided by Dr. R. Molday, University of British Columbia, Vancouver, Canada) diluted 1:500 and a mouse monoclonal antibody against synaptophysin (Chemicon) diluted 1:1000. Primary antibody incubation was performed over night at 4°C (see below).

The primary antibodies were localized with one of the following secondary antibodies. Fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG (Sigma), FITC-conjugated rabbit anti-goat IgG (Sigma), FITC-conjugated donkey anti-rabbit IgG Fab (Jackson Laboratories, West Grove, PA), or a Texas Red

conjugated donkey anti-rabbit IgG (Jackson Laboratories), each diluted 1:200 and applied for 45 min at room temperature. Sections were washed for 3x10 minutes in PBST between incubations and mounted using Vectashield antifade mounting medium (Vector Laboratories Inc., Burlingame, CA, USA). Occasional sections were mounted using Vectashield antifade mounting medium (Vector Laboratories) containing 4´6-diamidine-2-phenylindole-dihydrochloride (DAPI) to achieve nuclear staining. Both the primary and the secondary antiserum were diluted in PBST containing 1 % bovine serum albumin (BSA). Negative controls were performed by omission of the primary antibody.

Double-immunolabeled sections were pre-incubated with phosphate-buffered saline containing 0.25 % Triton X-100 (PBST) and 1 % bovine serum albumin (BSA; Sigma) for 15-20 min at room temperature. The sections were then incubated over night with recoverin antibodies at 4°C, followed by a Texas Red conjugated donkey anti-rabbit IgG (Jackson Laboratories, West Grove, PA, USA) diluted 1:200 for 45 min at room temperature. The sections were further processed with primary antibody against vimentin for 2-3 h at room temperature. The primary antibody was localized using a fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG (Sigma) diluted 1:200 and applied for 45 min at room temperature. Washes and mounting were performed as above.

The number of recoverin-positive cell bodies was counted in normal and cultured retinas, and data are presented as means±SEM. Statistical analysis was performed using Student´s *t* test.

3. Results

3.1 General observations

At P3 when the retinas were explanted, a neuroblastic layer (NBL) was clearly separated by a rudimentary inner plexiform layer from the ganglion cell layer (GCL)(Fig. 1A). At this stage, the outer NBL contained one or two rows of photoreceptor cells that were positive for rhodopsin and recoverin (Fig. 1 A-C). Some photoreceptor cells were clearly bipolarized with one distal process that projected to the retinal pigment epithelium (RPE) and one process that grew towards the inner retina (Fig. 1 B,C). Hu-immunolabeling showed that explants kept for 11 days *in vitro* differentiated into a laminated retina with separated nuclear and plexiform layers (Fig. 1 D), and grossly were morphologically comparable with P14 litter-mates (Fig. 1 E). A general observation was that *in vitro* specimens were reduced in thickness. Direct comparisons of cultured and P14 retinas showed a low cellular content of the GCL, possibly caused by the massive loss of ganglion cells (Rehen et al., 1996; Johansson et al., 2000; Manabe et al., 2002; Engelsberg et al., 2004). Reductions in thickness were evident in the INL and ONL, and the ON-region of the IPL was almost devoid of recoverin-positive bipolar cell terminals, which also contributed to a reduction of the IPL (see below). Further immunohistochemical examinations showed significant signs of remodeling in the inner retina of cultured specimens, including Müller cell hypotrophy and lateral branching in the inner retina, the formation of ectopic neuropil regions and sprouting of rhodopsin- and synaptophysin-positive photoreceptor cells.

3.2 Müller cells

To examine whether the culture condition influences the structural integrity of Müller cells, normal P14 and cultured retinas were immunostained for the intermediate

filaments vimentin and GFAP. Immunostaining for vimentin revealed various degrees of irregularities in the Müller cell cytoarchitecture in all cultured specimens. Radial Müller cell trunks appeared hypotrophic in comparison to P 14 retinas (compare Fig. 2 A and B). A considerable lateral Müller cell branching was also observed in the inner part of the IPL and GCL and in the inner nuclear layer (INL) of cultured retinas (Fig. 2 B). An irregularly array of fine lateral Müller cells processes was particularly evident in the ON-region of the IPL (Fig. 2 B, F). Expansion of Müller cell end foot processes at the vitreal border of the GCL was also observed *in vitro* (Fig. 2B, D). Finally, the radial processes of Müller cells in cultured retinas also exhibited an immunohistochemically detectable upregulation of GFAP (Fig. 2E). GFAP immunolabeling was evident from the vitreal border to the outer limiting membrane (data not shown), indicating that Müller cells may react to degenerative events initiated by the culture condition. Occasional GFAP-positive Müller cell processes were observed in the space between the photoreceptor outer segments and the RPE (data not shown).

3.3 Ectopic neuropil areas

Ectopic neuropil areas developed at various levels among cell bodies in the INL (Fig. 2 F,G). Analyses of several *in vitro* retinas from different cultures indicated that there was variability in the size and occurrence of the ectopic neuropil areas. In general, they exhibited an elongated morphology of varying length and a height of about one cell body diameter in thickness. Recoverin and vimentin immunolabeling showed the presence of both bipolar cell and lateral Müller cell processes in the ectopic neuropils, respectively (Fig. 2 B,F). The ectopic neuropils also exhibited immunolabeling for the presynaptic vesicle protein synaptophysin (Fig. 2 G). Judging from the variety of cell

processes and immunohistochemical markers, a growth of ectopic neuropil areas was associated with developing retinas maintained *in vitro.*

3.3 Cone bipolar cells

In cross-sections of control P14 retinas (Fig. 2 H), a dense network of recoverin-positive cone bipolar cell terminals was clearly distinguishable in the OFF-region and ON-regions (see Milam, Dacey, and Dizhoor, 1993) of the IPL. These terminals are derived from type 2 and type 8 cone bipolar cells, respectively (Euler and Wässle 1995). After 11 days *in vitro,* the number of recoverin-positive terminals in the ON-region was markedly reduced (Fig. 2 I). In each of the examined sections of cultured retinas, only single immunoreactive bipolar cell terminal expansions were observed in the ON-region (arrowed in Fig 2 D). Correspondingly, the density of recoverin-positive bipolar cell bodies in the inner nuclear layer (INL) of cultured retinas appeared different from the one observed in normal P14 specimens. The number of (mean±SEM) of recoverin immunoreactive cone bipolar cell bodies within 100 μm retina was 40.9 ±2.2 for P14 and 32.3±2.5 for cultured retinas (P=0.0015; n=5). The arborization pattern derived from immunopositive OFF cone bipolar cell terminal processes into the OFF-region was comparable to that observed in normal retinas (Fig. 2 H).

3.4 Photoreceptor cells

Synaptophysin immunolabeling showed similar distribution patterns in the IPL and OPL of cultured and P14 control retinas (Fig. 3 A-B). In addition, retina cultures showed neurites in the INL expressing synaptophysin immunolabeling. The neurites extended from the ONL and were found to project into various levels of the INL (Fig.

3 A). Occasional synaptophysin labeled neurites were also observed in the ectopic neuropil areas (Fig. 3 A). Because synaptophysin is found in photoreceptor cell terminals in the OPL (Kapfhammer, Christ, and Schwab, 1994; Dhingra, Ramamohan, and Raju, 1997), as well as in remodeling photoreceptor terminals in RP retinas (Fariss, Li, and Milam, 2000) and detached retinas (Fisher and Lewis, 2003), we interpret the synaptophysin-labeled neurites in cultured retinas as sprouting photoreceptor cells.

Rhodopsin antibodies most heavily label rod outer segments in cultured and P14 retinas (Fig. 3 C-D). Single rhodopsin-positive photoreceptor cell bodies were evident in the INL, which represent an early postnatal feature (Voyvodic, Burne, and Raff, 1995). Rod photoreceptors responded to culture by outer segment degeneration and/or abnormal development, which resulted in the redirection of the immunohistochemical expression for rhodopsin to the cell body. Consequently, intense rhodopsin labeling was found throughout the ONL after 11 days in culture (Fig. 3 C). In addition, sprouting rhodopsin-positive neurites extended from the ONL and passed through the OPL and projected into the INL. Occasional rhodopsin-positive neurites were found to project as far as the IPL (Fig. 3 C). Collectively, it appears that developing photoreceptors *in vitro* respond to the culture condition by sprouting neurites into the inner retina.

3.5 Amacrine cells

Two populations of well-known amacrine cell types, the cholinergic and the GABA-ergic subsets, connect to ganglion cells (Famiglietti, 1983; Chun, Kim, Oh, and Chung, 1999), and we investigated whether the *in vitro* condition would influence their structural integrity. Immunolabeling for choline acetyl transferase (ChAT) and

the vesicular acetylcholine transporter protein (VAChT) demonstrated 2 mirror-imaged cholinergic strata in the IPL of cultured retinas that were similar to the ones in normal retinas (Compare Figs. 4 A,C with Figs. 4 B,D). When compared to corresponding strata in P14 retinas, both strata exhibited a mild disorganized appearance in each of the examined *in vitro* specimens (Fig. 4A-D). Small clusters of displaced cholinergic amacrine cells were occasionally observed in the GCL (Fig. 4 A).

Immunolabeling for GABA in cultured specimens showed two major discrepancies from the pattern observed in P14 retinas. First, the stratification pattern of GABA-ergic terminals in the IPL was dramatically altered and was not evident (Fig. 4 E). Comparison with normal P14 retinas showed dense GABA immunoreactivity in strata 2 and 4 exhibited whereas the inner and outer margins of the IPL expressed weak GABA immunoreactivity (Fig. 4 F). Second, the density and localization of GABA-ergic amacrine cell in the *in vitro* GCL and INL were not directly comparable to *in vivo* specimens. There appeared to be a reduction of the GABA-ergic amacrine cells but it could not be concluded if they also were relocated within the INL.

4. Discussion

This and previous data show that explants of neonatal rat retina develop well *in vitro* and the retinal layering seen *in vivo* is evident. Except for rudimentary photoreceptor outer segments, immunohistochemical data demonstrate a gross morphological differentiation comparable with P14 litter-mates. However, the present study reports that structural changes develop in the explants including hypertrophy of Müller cell end foot processes forming glial scar-like structures at the vitreal aspect and signs of neuronal remodeling in the inner retina. The latter include the formation of ectopic

neuropil areas and reactive sprouting of photoreceptor neurites into the inner retina. Moreover, subsets of amacrine cells displayed subtle malformations in their IPL lamination patterns. The mechanism(s) whereby these changes occur are not known, but we suggest that photoreceptor and ganglion cell degenerations are possible contributory factors. The structural changes that arise in cultured retinas show morphological resemblance to neuronal remodeling demonstrated in RP retinas and experimentally detached retina.

4.1 Morphological considerations

Direct comparisons of cultured and normal P14 retinas demonstrate a reduction of retinal thickness after 11 days *in vitro.* These differences can be attributed to at least 2 mechanisms; decreased cellular content in the GCL and ONL as well as reduction of terminals in the IPL. During normal development of the IPL, the inner part of this layer undergoes relatively greater expansion after differentiation of the cholinergic strata and this expansion is related to the development of bipolar cell terminals (Reese et al., 2001). No such expansion of the inner retina was evident *in vitro.* We suggest that the failure of this expansion *in vitro* primarily is associated with ganglion cell death and the loss of cone bipolar cell terminals. Irregularities in Müller cell processes at the vitreal aspect may also contribute to the reduced GCL/IPL (see Germer, Kühnel, Grosche, Friedrich, Wolburg, Price, Reichenbach, and Mack, 1997). Moreover, the number of cell rows in the ONL is reduced by the experimentally induced photoreceptor cell death (Rehen et al., 1996; Pinzón-Duarte et al., 2000; Liljekvist-Larsson et al., 2003; Engelsberg et al., 2004), and thereby contributes to the thinning of the entire retina. Except for the redistibution of rhodopsin to the cell bodies and the presence of rudimentary outer segments, photoreceptor responses in

explanted retinas are unknown in detail. The damage to photoreceptors *in vitro* may be caused by various factors such as activated microglia, ischemia and loss of functional support normally provided by the retinal pigment epithelium (Merstch et al., 2001; Liljekvist-Larsson et al., 2003; Engelsberg et al., 2004).

4.2 Cellular responses to culture

Increased expression of intermediate filaments and morphological irregularities at the vitreal border was seen in Müller cells *in vitro,* as demonstrated by immunolabeling for GFAP and vimentin. Developmental studies show a constitutive expression for vimentin in rat Müller cells (Shaw and Weber, 1983) whereas the GFAP expression is low. Accumulation of GFAP in Müller cells has been demonstrated in response to injury, degenerative conditions and stress (reviewed by Lewis and Fisher, 2003). In the present study, morphological changes primarily appeared as lateral Müller cell branching and an immunohistochemical detection of GFAP in the radial processes. However, the *in vitro* responses were relatively rapid and developed in less then 11 days. In experimental retinal detachment, Müller cells responses are discernible within 1 day (Lewis, Matsumoto & Fisher, 1995).

Data from culture of intact adult retinas (Khodair, Zarbin, Townes-Anderson 2003), experimental detachment (Lewis et al., 1998; Fisher and Lewis, 2003) and inherited retinal degenerations in the *rd1* mouse (Strettoi, and Pignatelli, 2000; Fei, 2002; Strettoi, Porciatti, Falsini, Pignatelli, and Rossi, 2002; Strettoi, Pignatelli, Rossi, Porciatti, and Falsini, 2003) show remodeling in the cone and rod photoreceptors and pathways. Analyses of explanted retinas with antibodies to rhodopsin and synaptophysin demonstrated structural changes among photoreceptor cells. One unambiguous sign of photoreceptor degeneration and/or abnormal development *in*

vitro is represented by the redistribution of rhodopsin to the cell body (Fariss, Molday, Fisher, and Matsumoto, 1997). As in retinitis pigmentosa retinas (Fariss et al., 2000; Marc et al., 2003) and in detached and reattached retinas (Fisher and Lewis, 2003; Sethi, Lewis, Fisher, Leitner, Mann, Luthert, and Charteris, 2005), our data show that stressed immature photoreceptors sprout rhodopsin-positive neurites that extend into the IPL. This type of sprouting was further substantiated by the presence of synaptophysin immunoreactivity in presumptive photoreceptor terminals located among cell bodies in the INL. Thus, developing photoreceptors respond to culture and begin to degenerate, previously shown by the expression of different apoptotic markers (Engelsberg et al., 2004), and here by rhodopsin redistribution and photoreceptor sprouting. The latter appears to be an *in vitro* phenomenon since most photoreceptors were immature at explantation, and no substantial innervation of the IPL was observed (see Johnson, Williams, Cusato, and Reese, 1999).

As Pinzón-Duarte and colleagues (2000), we observed disturbed lamination patterns in the IPL of cultured retinas. Both cholinergic and GABA-ergic amacrine cells developed lamination patterns that appeared to be affected. It seems also reasonable to assume that the density of cholinergic and GABA-ergic amacrine cells was reduced *in vitro,* which is in accordance with previous studies of axotomized retina (Yamasaki, da Costa, Barbosa, and Hokoc, 1997; Cusato, Stagg, and Reese, 2001; Williams, Cusato, Raven, and Reese, 2001). Thus, one plausible mechanism for such changes in cultured retinas can be explained by the axotomy-induced loss of ganglion cells. The cholinergic amacrine cells are directly coupled to specific sets of ganglion cells (Famiglietti, 1983), while the GABA-ergic are in contact with recoverin-positive bipolar cells (Chun et al., 1999). Recent data suggest, however, that other mechanisms such as sensory deafferentation contribute to structural changes in

the IPL. Indeed, in human retinitis pigmentosa retinas the GABA lamination is disorder (Fariss et al., 2000) and the IPL also becomes fragmented (Jones et al., 2003). Fragmentation of the IPL and cellular migration are events that may be linked to Müller cell hypertrophy (Jones et al., 2003).

Analysis of several cultured retinas showed that recoverin- and vimentin processes projected towards and ramified within the ectopic neuropil areas. Such neuropil areas develop in cultured retinas and have been shown to express immunolabeling for the muscarinic acetylcholine receptor 2 (Johansson et al., 2000), which indicate the presence of post-synaptic structures. In the present study, synaptophysin labeling in ectopic neuropil regions suggests the presence of photoreceptor terminals in these areas. Whether the ectopic neuropil areas contain true synaptic contacts between appropriate neuronal elements and what mechanisms induce their development *in vitro* is currently unknown. Analysis of human retinas with photoreceptor degenerations (Jones et al., 2003), show that neuropil regions develop independently of early degenerative events and evolve after the degeneration of the outer nuclear layer.

In conclusion, we suggest that structural changes develop in cultured retinas and include evolution of novel neuropil areas, hypertrophy and expansion of Müller cell end foot processes and sprouting of photoreceptor cell terminals. Similar to the situation in RP retinas and detached retinas, glial and neuronal remodeling *in vitro* occurs in complex events over time. The *in vitro* model can be used to generate knowledge about these events and perhaps also allow experimental approaches to specifically control neuronal remodeling, photoreceptor sprouting and glial cell hypertrophy. Our data suggest that intact retinas used as treatment for inherited retinal dystrophies might have to be modified to promote functional host-graft integrations.

The first goal is to promote photoreceptor sprouting and reduce of glial remodeling and neuronal aberrant sprouting *in vitro* prior to transplantation.

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Figure legend

Figure 1.A-E. Micrographs of retinal layers at explantation (A-C), and after 11 days *in vitro* (D) compared to P14 retina (E). (A) The nuclear stain DAPI (blue) shows that the cellular are separated by an immature inner plexiform layer at P3. Rhodopsin immunoreactive photoreceptors (green) are localized to the outer part of the retina. (B-C) Developing photoreceptors express rhodopsin (B; green) and recoverin (C;red) immuoreactivity at P3. Note the polarized appearance of some cells. (D-E) Hu-immunolabeling (red) in cultured (D;C14) and control (E;P14) retinas demonstrating the reduced thickness of the IPL and GCL in cultured specimens. Abbreviations GCL ganglion cell layer, INL inner nuclear layer, IPL inner plexiform layer, NBL neuroblastic layer, ONL outer nuclear layer, OPL outer plexiform layer. Scale bars 10 μm.

Figure 2 A-I. Comparisons of cultured (C14) and control (P14) retinas demonstrating the glial and neuronal responses. (A) Micrograph of the P14 retina showing vimentin (green) labeled Müller cells. (B) Micrograph of corresponding area as in (A) of cultured retina, demonstrating vimentin labeling (green) in hypertrophized trunks of Müller cells. Note also the lateral Müller cell branching in the IPL and INL. (C) In the P14 retina, recoverin-positive (red) cone bipolar cells terminals are seen in the ON- and OFF-region. Distinctly vimentin (green) immunoreactive Müller cell end feet are evident at the vitreal aspect. (D) In retina maintained for 11 days *in vitro,* vimentin immunoreactivity is found in the end foot and lateral processes of the Müller cells. (E) In cultured retinas, Müller cells upregulate GFAP immuoreactivity in the end foot and radial processes of. Small GFAP- positive lateral processes (arrow) are seen in the

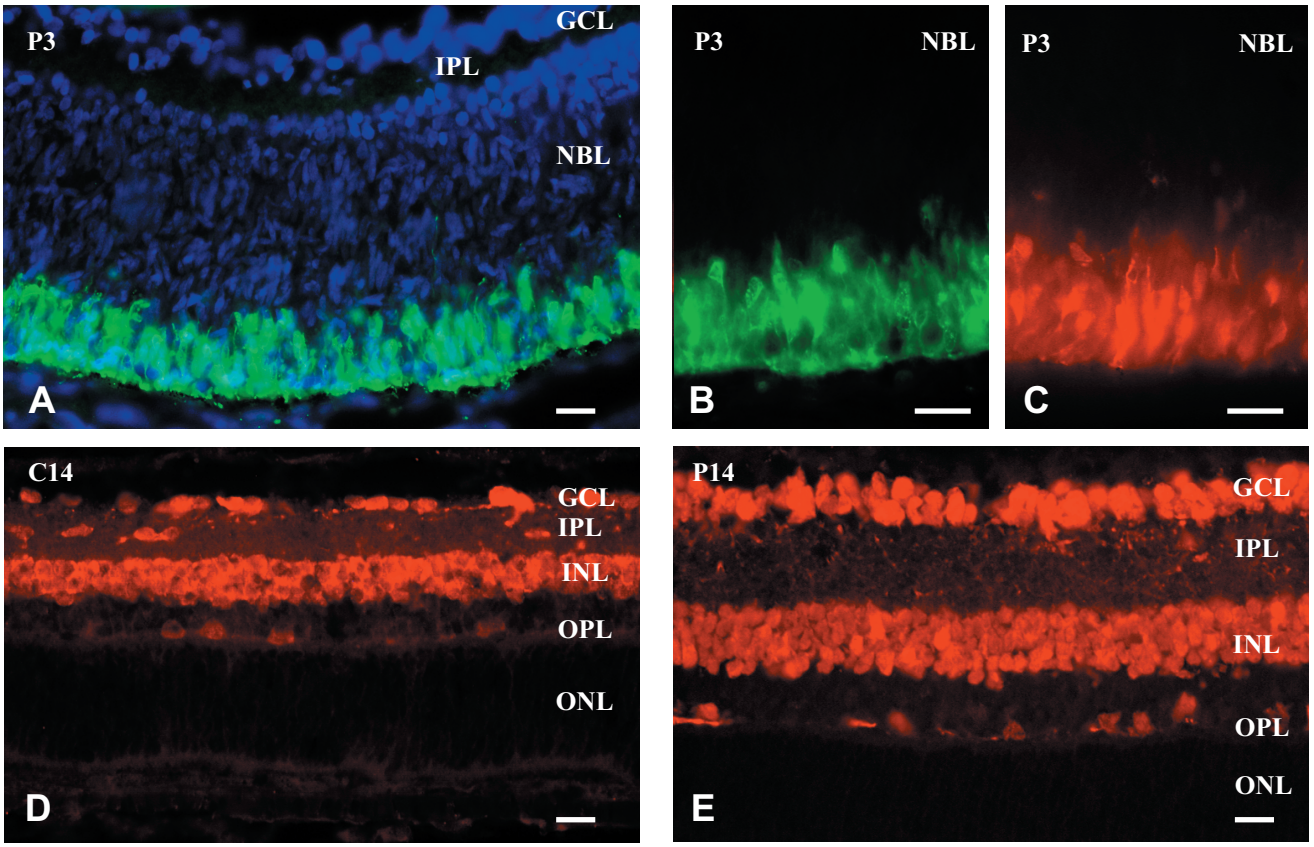
IPL. (F-G) Examples of ectopic neuropil areas in cultured retinas. (F) Both recoverin (red) and vimentin (green) immunolabeled processes (arrow) are evident the neuropil. (G) The ectopic neuropils also exhibit immunolabeling for synaptophysin. (H) In P14 retinas cone bipolar terminals labeled with anti-recoverin (red) extend into ON- and OFF-regions. (I) The ON-region of cultured retinas is devoid of cone bipolar cell terminals. Notice the bipolar cell innervation of the OFF-region. Abbreviations as in Fig. 1. Scale bars 10 μm.

Figure 3 A-D. Micrographs illustrating photoreceptor responses in cultured retinas. (A) Synaptophysin immunoreactive processes (small arrows) are present in the INL after 11 days *in vitro.* Synaptophysin immunoreactivity is also evident in the ectopic neuropil areas (large arrows) and the OPL (asterisk). (B) At P14, synaptophysin immunoreactivity is restricted to the plexiform layers. Part of the heavily labeled OPL (asterisks) is indicated. (C) Sprouting of rhodopsin immunoreactive terminals into the INL in cultured retinas. Some terminals (large arrow in insert) approach the IPL. (C) Rhodopsin-positive cell bodies are evident in the INL of cultured retinas (small arrows). Notice differences in the intensity of rhodopsin labeling in the ONL of cultured (C) and P14 retina (D). Abbreviations as in Fig. 1. Scale bars 10 μm.

Figure 4 A-F. Photomicrographs of retinal sections showing the stratification pattern of cholinergic and GABA-ergic amacrine cells in cultured and litter-matched P14 retinas. The cholinergic amacrine cells show similar distribution pattern in cultured (A, C) and normal P14 retinas (B, D). (A, C) Notice the disorganized appearance of the ChAT and VAChT labeled strata in cultured specimens. (E, F) The GABA-ergic

stratification pattern appears disorganized *in vitro* (E) in comparison to the one in the IPL of P14 retinas (F). Abbreviations as in Fig. 1. Scale bars 10 μm.

Figure


P3
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A
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B
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Figure

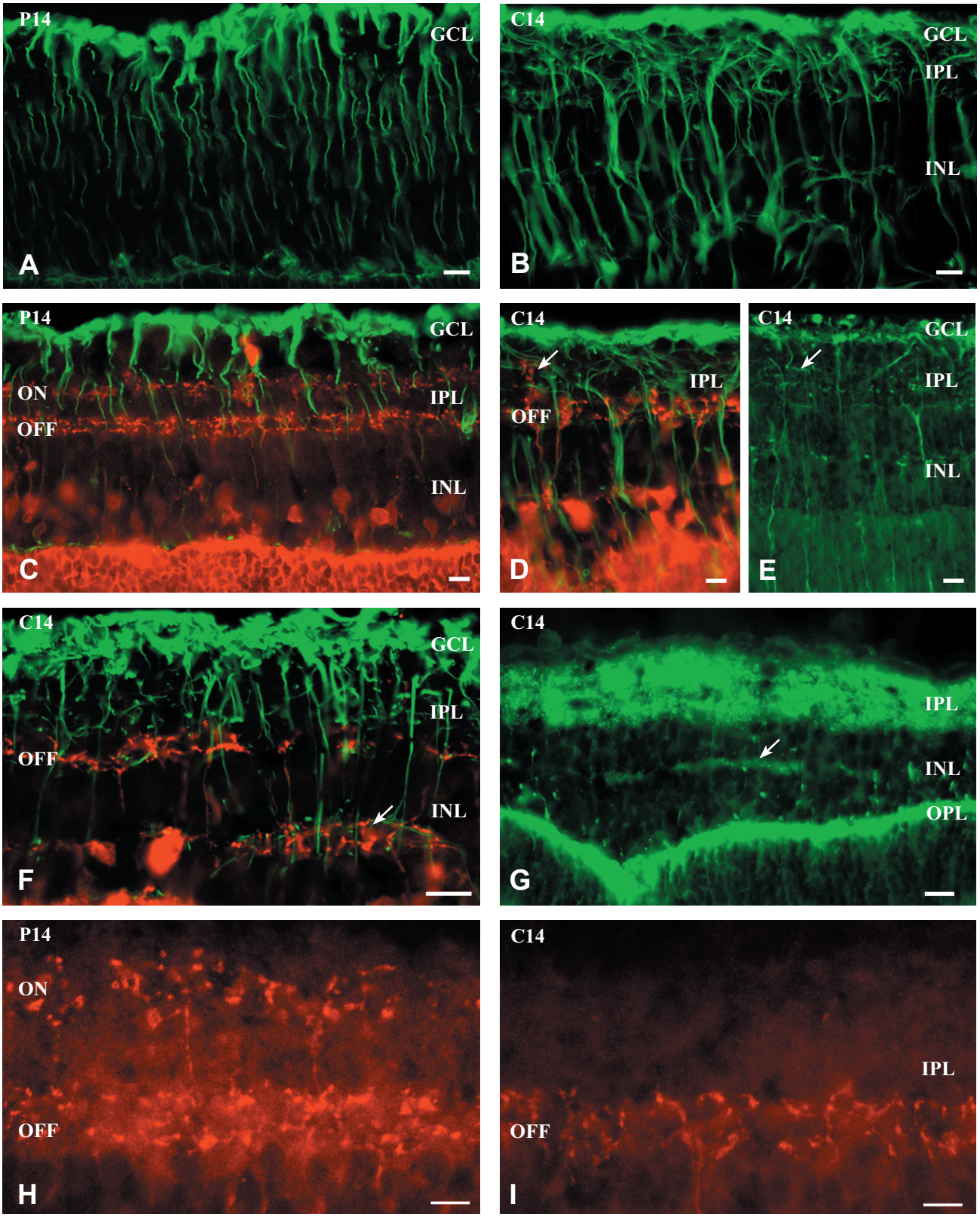
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Figure 3

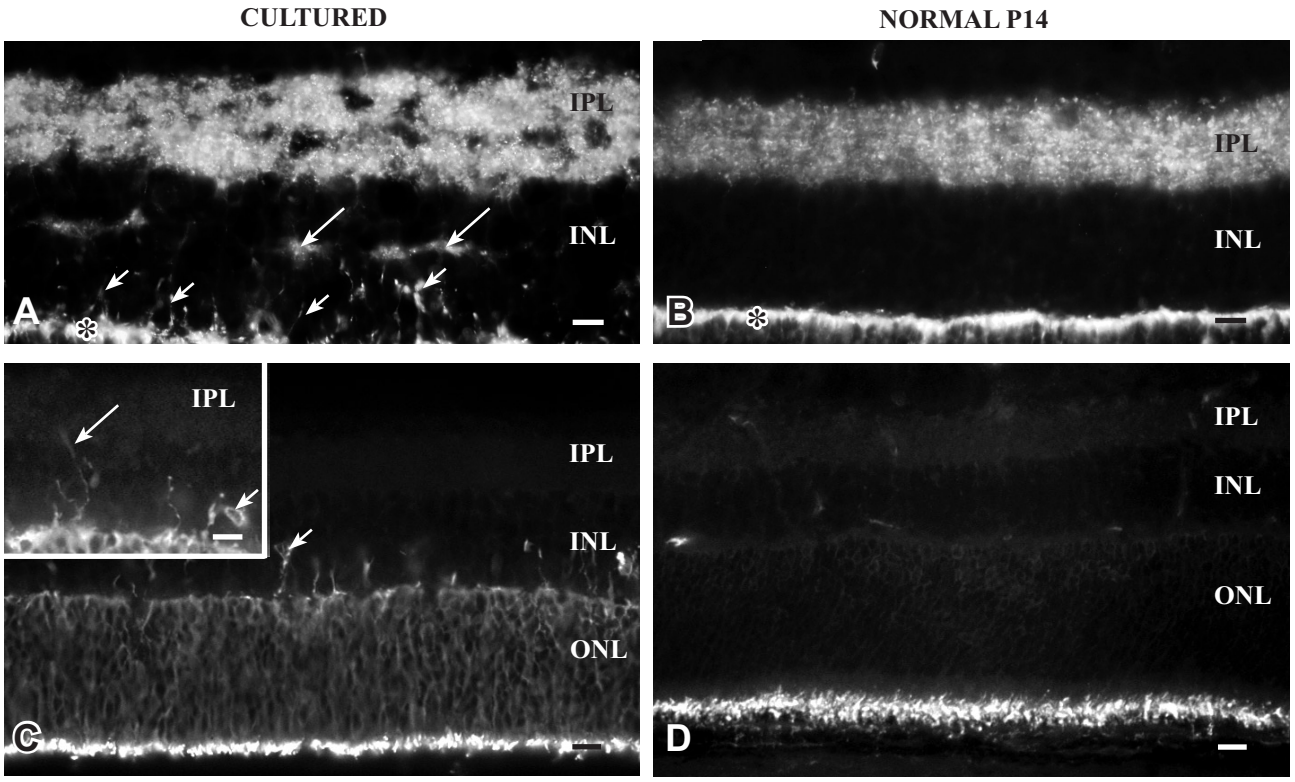
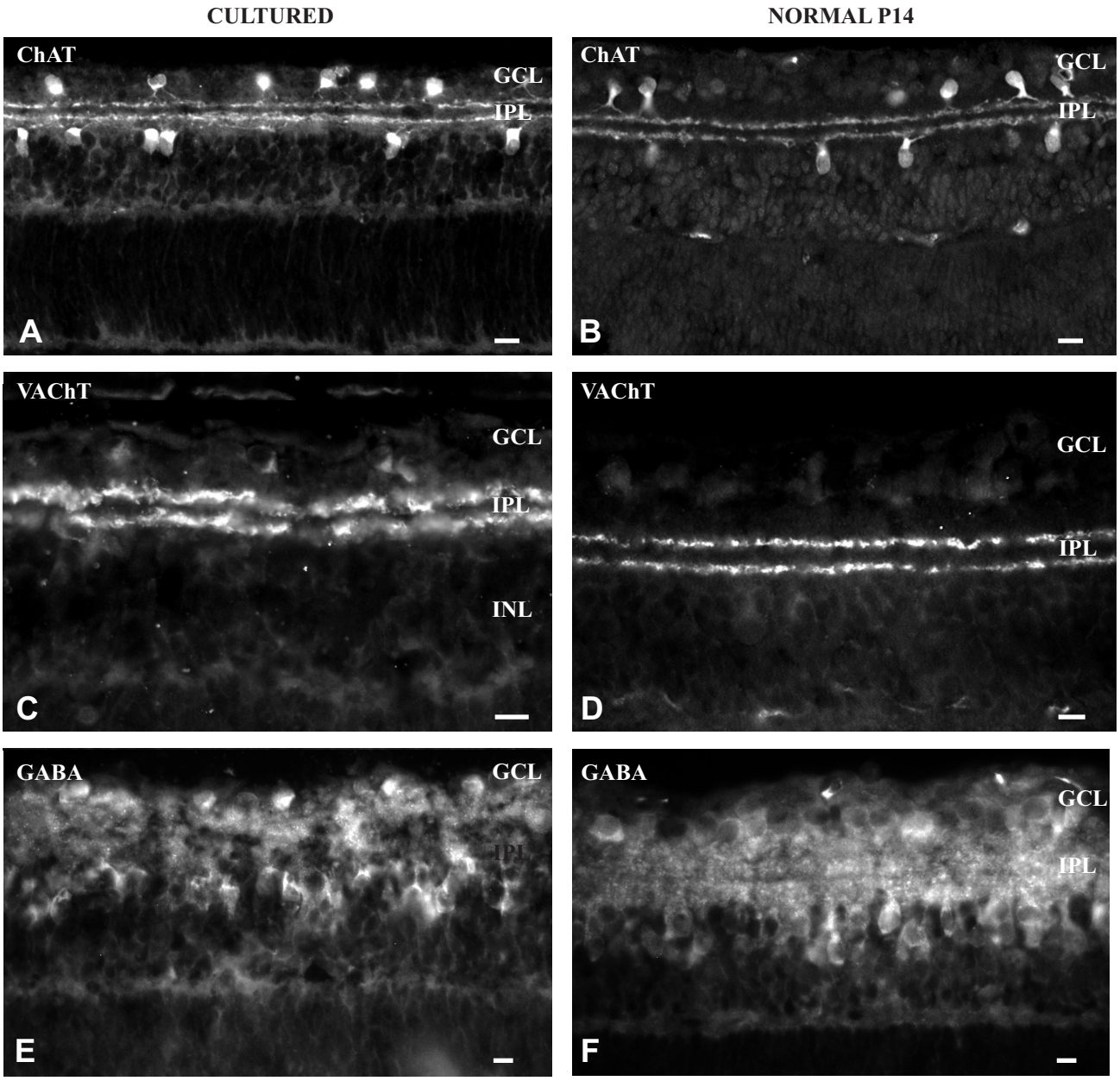


Figure 4


CULTURED
NORMAL P14
ChAT
GGL
IPL
A
ChAT
GCL
IPL
B
VAChT
GCL
IPL
INL
C
VAChT
GCL
IPL
D
GABA
GCL
IPL
E
GABA
GCL
IPL
F