Connexin Immunoreactivity in Glial Cells of the Rat Retina

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ABSTRACT

The rat retina contains two types of macroglial cells, Müller cells, radial glial cells that are the principal macroglial cells of vertebrate retinas, and astrocytes associated with the surface vasculature. In addition to the often-described gap-junctional coupling between astrocytes, coupling also occurs between astrocytes and Müller cells. Immunohistochemistry and confocal microscopy were used to identify connexins in the retinas of pigmented rats. Several antibodies directed against connexin43 stained astrocytes, identified using antibodies directed against glial fibrillary acidic protein (GFAP). In addition, two connexin43 antibodies stained Müller cells, identified with antibodies directed against S100 or glutamine synthetase. Connexin30-immunoreactive puncta were confined to the vitreal surface of the retina and colocalized with GFAP-immunoreactive astrocyte processes. Connexin45 immunoreactivity was associated with both astrocytes and Müller cells. We conclude that retinal glial cells express multiple connexins, and the patterns of immunostaining that we observe in this study are consistent with the expression of connexin30, -43, and possibly -45 by astrocytes and the expression of connexin43 and -45 by Müller cells. As gap-junction channels may be formed by both homotypic and heterotypic hemichannels, and the hemichannels may themselves be homomeric or heteromeric, there exists a multitude of possible gap-junction channels that could underlie the homotypic coupling between retinal astrocytes and the heterotypic coupling between astrocytes and Müller cells.


Indexing terms: gap junction; immunohistochemistry; immunostaining; astrocyte; Müller cell

Gap junctions may underlie a dynamic and complex network for signaling between glial cells. In addition to the often-described gap-junctional coupling between astrocytes (Tani et al., 1973; Yamamoto et al., 1990b; Dermietzel et al., 1991; Giaume et al., 1991; Lee et al., 1994; Ransom, 1995), coupling has been observed between astrocytes and oligodendrocytes (Ransom and Kettenmann, 1990; Robinson et al., 1993; Ochalski et al., 1997) and between astrocytes and Müller glial cells in the retina (Robinson et al., 1993; Zahn and Newman, 1997). Gap junction channels provide a direct route for the intercellular exchange of ions and small metabolites and have been proposed to have a role in K⁺ buffering (Mobbs et al., 1988), exchange of glucose metabolites (Tabernero et al., 1996; Giaume et al., 1997), and propagation of intercellular Ca²⁺ waves (Charles et al., 1992, 1993).

Each gap-junction channel is formed by the docking of two hemichannels, contributed by each cell of the coupled pair. These hemichannels, or “connexons,” are hexamers composed of members of the connexin family of proteins (Beyer et al., 1987, 1990). Fifteen connexins have been identified thus far in mammals (Güldenagel et al., 2000).

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Gap junctions thus constitute a family of channels that may vary in their selective permeabilities, voltage sensitivities, rectification properties, and modes of regulation (Mills and Massey, 1995; Kumar, 1999; Beyer et al., 2000; Dermietzel et al., 2000a; Nicholson et al., 2000; White and Bruzzone, 2000).

The retina is a particularly good model system for studying the nature and regulation of gap-junctional communication between glial cells in situ. Vascularized mammalian retinas contain two major classes of macroglial cells, astrocytes and Müller cells. Astrocytes are present at the vitreal surface of the retina, and their processes ramify mainly in two dimensions. Müller cells are radial glia that span the depth of the retina. Both cell types are easily identifiable and accessible for electrophysiological recording and imaging studies in acutely isolated retinas (Robinson et al., 1993; Clark and Mobbs, 1994; Zahs and Newman, 1997). Gap junctions between retinal astrocytes and between astrocytes and Müller cells have been demonstrated through the intercellular spread of gap junction-permeant tracers. Gap junctions between astrocytes are permeable to both the fluorescent tracer Lucifer yellow and the biotin derivatives neurobiotin and biocytin, whereas the gap junctions between astrocytes and Müller cells are permeable primarily (rabbit, Robinson et al., 1995) or exclusively (rat, Zahs and Newman, 1997) to the biotin derivatives. Furthermore, in both species, tracer has been observed to spread only in the direction of astrocyte to Müller cell (Robinson et al., 1993; Zahs and Newman, 1997).

The apparent asymmetry of the coupling between retinal astrocytes and Müller cells could arise if the hemichannels contributed by astrocytes and Müller cells are formed by different connexins (Plagg-Newton and Loewenstein, 1980). The present study was undertaken to determine which connexins are present in retinal glia. Immunostaining coupled with confocal microscopy was used to identify several connexin proteins in the rat retina. Connexin43 (Cx43), connexin30 (Cx30), and connexin45 (Cx45) immunoreactivities were found to colocalize with astrocyte processes at the retinal surface. An antibody that recognizes Cx43 only when serine 368 is not phosphorylated (Nagy et al., 1997; Cruciani and Mikalsen, 1999) was found to stain Müller cells, and Cx45 immunoreactivity was observed to colocalize with Müller cell markers throughout the depth of the retina.

In situ fixation

Rats were anesthetized and transcardially perfused with Ringer’s solution as described above. One eye was removed and the retina isolated as described, while the rat continued to be perfused with 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4. The second eye was then removed from the animal, and the retina was dissected into PBS, then postfixed overnight in 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4, at 4°C. We were thus able to compare the pattern of immunostaining in the retina fixed in situ with the pattern of immunostaining in the paraformaldehyde-fixed, isolated retina from the same animal.

Immunostaining of retinal whole mounts

Immunostaining was conducted using a panel of antibodies directed against specific connexins. These antibodies were listed in Table 1. Most retinas were doubly immunostained with an anti-connexin antibody and an antibody directed against one of the following glial cell markers: glial fibrillary acidic protein (GFAP), to label retinal astrocytes (mouse anti-GFAP, Chemicon, Temecula, CA, MAB360, diluted 1:200); or rabbit anti-GFAP, Chemicon AB5040, diluted 1:50; glutamine synthetase (GS), to label Müller cells (mouse anti-GS, Chemicon MAB302, diluted 1:200; or goat anti-GS, Santa Cruz Biotechnology, Santa Cruz, CA, C-20, diluted 1:200); or S100, to label both astrocytes and Müller cells (rabbit anti-S100 [β-homodimer], Dako, Carpineteria, CA, Z0311, 10 μg/ml; or mouse anti-S100, Chemicon, MAB079-1, 10 μg/ml). Secondary antibodies were obtained from Jackson Immunore-
from the same retinas as the corresponding experimental samples. For each antibody, immunostaining was performed on at least three samples obtained from different animals on different days.

**Retinal sections**

Two of the antibodies (Zymed 13-8300, monoclonal anti-Cx43; Chemicon MAB3101, monoclonal anti-Cx45) were also tested on cryosections of retina. Lethal injections of sodium pentobarbital were administered to two rats, and the eyes were enucleated and immersion fixed for 4 hours in 4% paraformaldehyde in 0.1 M PB at 4°C. The eyes were then rinsed three times in PBS, the cornea and lens were removed, and the resulting eyeucps were cryoprotected in 30% sucrose in PB and embedded in a mixture of OCT compound (Miles Scientific, Naperville, IL) and Aqua-Mount (Lerner Laboratories, Pittsburgh, PA) (Jones et al., 1986). Twelve-micrometer-thick sections were cut on a cryostat.

Sections were doubly immunostained using Dako rabbit anti-S100 (10 µg/ml) or mouse anti-Cx43 (Zymed 13-8300, 5 µg/ml) or mouse anti-Cx45 (Chemicon MAB3101, 5 µg/ml). Sections were gently rinsed four times with PBS, then incubated for 1 hour at room temperature with 10% normal donkey serum diluted in PBS with 1% Triton X-100. This blocking solution was then removed, and the sections were incubated for 16 hours at 4°C with primary antibodies diluted in the same buffer as was used for the retinal whole mounts. Retinas were then rinsed four times with PBS and incubated with fluores-
TABLE 2. Summary of Results

<table>
<thead>
<tr>
<th>Immunohistochemistry</th>
<th>Control</th>
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</thead>
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<tr>
<td>Connexin26</td>
<td>Diffuse staining of astrocyte processes</td>
</tr>
<tr>
<td>Zymed 71-0500</td>
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</tr>
<tr>
<td>Zymed 13-8100</td>
<td>No staining</td>
</tr>
<tr>
<td>Connexin30</td>
<td>No staining</td>
</tr>
<tr>
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</tr>
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<tr>
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</tr>
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<tr>
<td>Zymed 71-0700, lot 90647439</td>
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</tr>
<tr>
<td>KacCx43</td>
<td>No primary antibody</td>
</tr>
<tr>
<td>Zymed 13-8300</td>
<td>No primary antibody</td>
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<tr>
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<td>No primary antibody</td>
</tr>
<tr>
<td>Chemicon AB1745</td>
<td>No primary antibody</td>
</tr>
<tr>
<td>Chemicon MAB3100</td>
<td>Dendrites in IPL, possibly Müller cells, inconsistent</td>
</tr>
<tr>
<td>Chemicon MAB3101</td>
<td>Müller cells, astrocytes, possibly dendrites in IPL</td>
</tr>
</tbody>
</table>

1Demonstrated cross-reactivity with connexin30 (Nagy et al., 1999).
2Concentration of peptides (weight/volume) expressed relative to concentrations of antibodies.
3Demonstrated cross-reactivity with connexin43 (Coppen et al., 1998).

Confocal microscopy

Immunostained retinas were imaged with a Leica TSM laser scanning confocal microscope. For a detailed examination of the staining pattern in particular layers of the retina, retinal whole mounts were used. Optical sections parallel to the retinal surface were collected using a 100× oil-immersion objective. Five to twenty serial confocal images were collected at 0.2 μm intervals within each retinal layer. When collected as 512 pixel × 512 pixel, eight-bit grayscale images, these images had a resolution of 0.2 μm/pixel. As a result of variations in antibody penetration and light scattering, the intensities of the fluorescence signals obtained in each layer of the retinal whole mounts may not accurately reflect the relative amounts of antigens in each layer. Because it was not possible to make quantitative comparisons between layers, the settings for the laser and photomultiplier tubes (PMTs) were optimized for each retinal layer.

Retinal sections were scanned using a 50× oil-immersion objective. Images were collected as 512 pixel × 512 pixel, eight-bit gray-scale images, which resulted in a resolution of 0.4 μm/pixel. Images were collected at 0.4 μm intervals through the thickness of the section.

Simultaneous detection of FITC and Texas red or Cy3 was accomplished using 488 nm/568 nm excitation from an ArKr laser, with 530/30 nm bandpass (FITC) and 665 nm (Texas red) or 590 nm (Cy3) longpass emission filters. Control samples were scanned using the same settings for the laser and PMTs as were used to scan the corresponding experimental samples.

Image reconstruction

The figures presented in Results were created using Adobe Photoshop running on a PC, after confocal images were first processed using MetaMorph software (Universal Imaging, West Chester, PA). To illustrate the pattern of staining within a retinal layer, a series of confocal images spanning 1–2 μm was projected onto a single plane by assigning to each pixel in the projection the intensity of the brightest pixel at that X-Y location from among those in the stack of images. To illustrate the pattern of staining in the retinal sections, MetaMorph was similarly used to project stacks of confocal images (spanning 10 μm) onto a single plane. For side-by-side comparisons of immunostained retinas and their corresponding controls, the MetaMorph projections for the two samples were pasted into a single image within Photoshop, and adjustments to brightness and contrast were performed simultaneously on the two samples. It should be noted that, for the images depicting the staining within each retinal layer, the brightness and contrast were adjusted for each layer separately. These figures therefore do not reflect relative amounts of staining in those layers. However, the images of the retinal sections provide an accurate representation of the relative amounts of staining through the depth of the retina.

Western blotting

Male Long-Evans rats (250–400 g) were killed with an overdose of sodium pentobarbital (150 mg/kg) administered intraperitoneally. Retinas, brain (cerebral cortex), heart (ventricles), and liver were rapidly dissected, washed with PBS, and homogenized in sample buffer containing 320 mM sucrose, 0.2 mM phenylmethylsulfonyl fluoride, peptstatin A (1 μg/ml), leupeptin (1 μg/ml), and benzamidine (100 μg/ml; all protease inhibitors from Sigma). Homogenates were centrifuged for 5 minutes at 2,000g (4°C) to remove insoluble material. The supernatant was then centrifuged at 100,000g (4°C) to obtain the
crude membrane fraction, which was then washed once in the sample buffer. The protein concentration in the crude membrane fraction was determined using the Coomassie Plus Protein Assay Reagent Kit (Pierce, Rockford IL), following the manufacturer's instructions. Bovine serum albumin was used as the standard. Membranes were stored at −80°C.

Proteins in the membrane preparations from each tissue of interest were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE; 4–20% gradient gel; Invitrogen, Carlsbad, CA) under reducing conditions and electrophoretically transferred to a 0.2 μm polyvinylidene difluoride membrane (Invitrogen). Blots were incubated with 5% powdered milk in PBS with 0.2% Tween-20 for 1 hour at room temperature to block nonspecific binding of the antibodies. After they were rinsed in PBS, blots were incubated overnight at 4°C with primary antibody diluted in PBS/0.2% Tween-20/1% powdered milk. Primary antibodies were used at the following dilutions: rabbit anti-Cx30 (Zymed; 71-2200, lot 00460029, 1:1,000 = 0.5 μg/ml), rabbit anti-Cx43 (Zymed; 71-0700, lot 80140147, 1:1,000 = 0.5 μg/ml; and lot 90647439, 1:10,000 = 50 ng/ml), mouse anti-Cx30 (Zymed; 13-8300, 1:1,000 = 1 μg/ml), and mouse anti-Cx43 (Chemicon; MAB3101, 1:1,000 = 1 μg/ml). For peptide preabsorption controls, prior to addition to the blots, primary antibodies were incubated for 2–4 hours at room temperature with a 5× concentration (weight/volume) of the peptide against which the antibody was generated. After the primary antibody incubation, blots were rinsed for 3 × 15 min in PBS/0.2% Tween-20/5% powdered milk, then incubated with secondary antibody for 1 hour at room temperature (horseradish peroxidase-conjugated donkey anti-mouse 1:10,000 or donkey anti-rabbit 1:3,000; Jackson Immunoresearch; diluted in PBS/0.2% Tween-20/1% powdered milk). After being rinsed with PBS, blots were developed using a chemiluminescence method (ECL: Amersham, Piscataway, NJ; or LumiLight-Plus: Roche Diagnostics, Indianapolis, IN).

In some cases, after antibody incubation and development, blots were stripped of antibodies and reprobed. Blots were incubated in stripping buffer (100 mM 2-mercaptoethanol, 2% SDS, 62.5 mM Tris-HCl, pH 6.7) for 45 minutes at 70°C. Removal of the antibodies was confirmed by developing the exposed blots with chemiluminescence reagents. In all cases, there was no detectable signal after stripping, indicating that the secondary antibodies, at least, had been removed. Blots were rinsed for 3 × 10 min with PBS-Tween and then probed with another primary antibody.

**RESULTS**

The results obtained with the various anti-connexin antibodies are summarized in Table 2, and patterns of immunostaining are described in detail below.

**Cx30**

Cx30-immunoreactive (-IR) puncta colocalized with GFAP-IR astrocyte processes (see Fig. 2A,B). Although we did not subject the images to quantitative analysis, it was our impression that the Cx30 immunoreactivity was localized primarily on the distal processes of astrocytes, where they contacted blood vessels. There were no qualitative differences in staining between retinas fixed in situ and acutely isolated retinas. Peptide preabsorption eliminated staining with this antibody (not shown).

On Western blots, this antibody stained a band at approximately 30 kDa in membrane preparations from brain as well as a fainter band at approximately 50 kDa. A single band, at approximately 33–34 kDa, was stained in membrane preparations from the retina (Fig. 1D). The differences in electrophoretic mobility of the Cx30-IR bands from brain and retina might represent differences in posttranslational modification of Cx30 in the two tissues or even the differential expression of two Cx30 transcripts (Dahl et al., 1996).

**Cx43**

Four polyclonal and two monoclonal antibodies directed against Cx43 were used to stain retinal whole mounts. Four of the antibodies (Zymed 71-0700, lots 50826441 and 80140147; KAc-Cx43; Chemicon MAB3068) yielded discrete immunoreactive puncta that were located primarily within the nerve fiber layer at the vitreal surface of the retina. In retinas doubly immunostained with an antibody directed against GFAP, the Cx43-IR puncta were located along GFAP-IR astrocyte processes (Fig. 2C,D). Cx43-IR puncta in the retinal ganglion cell (RGC) layer and inner plexiform layer (IPL) were associated with the occasional astrocyte processes that descended into these layers. There were no qualitative differences in staining between retinas fixed in situ and acutely isolated retinas.

The Zymed monoclonal antibody (13-8300) directed against Cx43 yielded a very different pattern of staining. In ethanol-fixed tissue, diffuse staining of Müller cells was observed (not shown). In paraformaldehyde-fixed retinas, punctate immunoreactivity was present throughout the depth of the retina. This Cx43 immunoreactivity was localized to Müller cells, identified in retinas doubly labeled with 13-8300 and antibodies directed against either S100 or glutamine synthetase. In the sample illustrated in Figure 3, a polyclonal anti-S100 labeled both astrocytes and Müller cell endfeet at the vitreal surface of the retina. Cx43 immunoreactivity was observed primarily over the Müller cells, distinguishable from the astrocytes by their fainter labeling with the anti-S100. Within the RGC layer, immunoreactive puncta were observed in S100-IR Müller cell processes, located between neuronal somata. Punctate Cx43 immunoreactivity was observed throughout the inner plexiform layer. Within the IPL, the proximal stalks of Müller cells that traverse this layer were strongly immunoreactive for S100. However, there was also fainter, more diffuse S100 immunoreactivity that may represent the fine side branches of Müller cells that wrap the synapses in the IPL (Newman and Reichenbach, 1996). Since S100 may be secreted by glial cells, it is also possible that some of the diffuse S100 immunoreactivity was extracellular (Shashoua et al., 1984; Van Eldik and Zimmer, 1987). In the inner portion of the inner nuclear layer (INL), punctate staining was observed in S100-IR profiles located between unstained neuronal somata. Within the middle portion of the INL, Cx43 immunoreactivity was observed primarily over S100-IR Müller cell processes, although occasionally Cx43-IR puncta were observed at the borders between S100-IR Müller cell somata. Because it was not possible to distinguish individual Müller cell processes in these samples, we were unable to determine whether Cx43 immunoreactivity occurred within or between pro-
cesses. This pattern persisted in the outer portion of the INL. While Cx43-IR puncta were observed in the outer plexiform layer, we could not clearly distinguish the processes of Müller cells in this layer. Within the outer nuclear layer (ONL), S100-IR Müller cell processes surrounded the photoreceptor cell bodies, and Cx43-IR profiles were again located over these Müller cell processes. In retinas doubly labeled with 13-8300 and antibodies directed against GFAP, some Cx43-IR puncta were located along astrocyte processes, although the majority of the Cx43 immunoreactivity at the retinal surface did not colocalize with astrocytes (Fig. 2G). There were no qualitative differences in staining between retinas fixed in situ and acutely isolated retinas. Peptide preabsorption eliminated staining with this antibody.

In radial sections of the retina (Fig. 3G–K), antibody 13-8300 strongly labeled the endfeet and the proximal stalks of Müller cells. Additional Cx43 immunoreactivity was associated with S100-IR Müller cell somata and processes throughout the depth of the retina, consistent with the pattern of staining seen in the retinal whole mounts.

Finally, a third lot of the polyclonal Zymed 71-0700 (lot 90647439) yielded punctate immunoreactivity throughout the depth of the retina. At the retinal surfaces, this Cx43 immunoreactivity colocalized with GFAP-IR astrocyte processes (Fig. 2D). In other retinal layers, Cx43 immu-

Fig. 1. Western blots. Proteins in the membrane preparations from each tissue of interest were separated by SDS-PAGE under reducing conditions, transferred to PVDF membranes, and probed with various anti-connexin antibodies, as described in Materials and Methods. A: Polyclonal rabbit anti-rat Cx43 (Zymed 71-0700, lot 80140147). Because of the large difference in the intensity of bands from brain and heart compared with retina, the lanes from brain and heart are shown from a film exposed for a shorter period than the film used to show the bands in retina. B: Polyclonal rabbit anti-rat Cx43 (Zymed 71-0700, lot 90647439). C: Monoclonal mouse anti-rat Cx43 (Zymed 13-8300). D: Polyclonal rabbit anti-mouse Cx30 (Zymed 71-2200, lot 00466029). E: Monoclonal mouse anti-human Cx45 (Chemicon MAB3101). Positions of molecular weight markers are shown to the left of each blot. B, brain (cerebral cortex); H, heart (ventricles); R, retina; subscripts indicate amount of protein (in micrograms) loaded in each lane.
noreactivity was localized to Müller cells, identified with either anti-S100 or anti-glutamine synthetase (not shown), and appeared very similar to the immunoreactivity observed with the Zymed 13-8300.

Western blots were used to assess the specificity of the antibodies that gave the most robust staining. Although they produced different patterns of immunostaining in retinal whole mounts, the two polyclonal antibodies tested (Zymed 71-0700, lots 80140147 and 90647439) yielded similar results on Western blots (Fig. 1A,B). In brain and retina, both antibodies detected bands at approximately 43 kDa and approximately 32 kDa. The latter band may represent a Cx43 proteolysis product; bands of similar size have been reported in Western blots of brain and retina (Giblin and Christensen, 1997) and heart (Manjunath et al., 1987; Hofer and Dermietzel, 1998) with the use of other anti-Cx43 antibodies. Similar bands were detected in blots from heart, except that, in the heart, these antibodies detected at least a doublet at approximately 43 kDa. Such doublets, and even triplets, are commonly detected by anti-Cx43 antibodies in blots from heart and represent the presence of (multiply) phosphorylated forms of Cx43 (Musli and Goodenough, 1991; Laird et al., 1991). The absence of the bands representing the (multiply) phosphorylated forms of Cx43 in samples from retina and brain may be due to a greater susceptibility of Cx43 to post-mortem dephosphorylation in neural tissues than in heart in our preparations (Hossain et al., 1994b). The monoclonal anti-Cx43 (Zymed 13-8300) stained a band at approximately 43 kDa in samples from brain and retina as well as higher molecular weight species that may represent connexin multimers (Fig. 1C). This antibody has been reported to recognize Cx43 only when serine 368 is not phosphorylated (Nagy et al., 1997; Cruciani and Mikalsen, 1999).

**Cx32**

Two antibodies directed against Cx32 (Zymed polyclonal antibody 71-0600 and Chemicon monoclonal antibody MAB3069) yielded punctate staining throughout the depth of the retina (not shown). However, because these antibodies yielded a pattern of immunostaining in retinas from Cx32-null mice that was indistinguishable from that in rat retinas, it was concluded that these antibodies do not specifically immunostain Cx32 in the retina.

**Cx45**

Initial experiments using a polyclonal antibody directed against Cx45 (Chemicon AB1742) yielded punctate staining throughout the depth of the retina (not shown). This antibody was subsequently shown to cross-react with Cx43 (Coppen et al., 1998). Later experiments using a second polyclonal antiserum (Chemicon AB1745) generated against a synthetic peptide corresponding to amino acids 354–367 of human Cx45, which does not possess sequence homology with Cx43 (Coppen et al., 1998), yielded a similar pattern of staining: immunoreactive puncta were observed throughout the depth of the retina, and these Cx45-IR puncta were primarily associated with Müller cells identified using either anti-S100 or anti-glutamine synthetase (not shown). Peptide preabsorption greatly reduced, but did not completely eliminate, staining with this antibody. Finally, punctate immunoreactivity that largely colocalized with Müller cell markers was obtained with a monoclonal antibody (Chemicon MAB3101) directed against amino acids 354–367 of human Cx45 (Fig. 4). Pronounced Cx45 immunoreactivity was found in the inner retina. Cx45-IR puncta were observed over astrocytes at the vitreal surface in retinas doubly labeled with anti-S100 (Fig. 4A) and over Müller cell processes in the RGC layer (Fig. 4B). In the IPL, some Cx45 immunoreactivity occurred over the proximal stalks of Müller cells, identified by heavy S100 immunoreactivity. However, as with Cx43 immunoreactivity in the IPL, it was not possible to determine whether the majority of Cx45-IR profiles in this layer was associated with the fine processes of Müller cells. In the middle of the INL, Cx45-IR puncta were located over S100-IR Müller cell somata and processes (Fig. 4D). In addition, linear arrays of immunoreactive puncta in the innermost region of the INL may represent staining of neuronal processes (Fig. 4C). At the outer border of the INL (Figs. 4E, 5), Cx45 immunoreactivity occurred over S100-IR Müller cell processes surrounding horizontal cells. Finally, Cx45 immunoreactivity occurred over Müller cell processes surrounding the somata of photoreceptors in the ONL (Fig. 4F). Peptide preabsorption eliminated staining with this antibody.

Staining of radial sections of the retina with MAB3101 confirmed the laminar distribution of connexin immunoreactivity observed in the whole mounts. However, the radial sections proved to be less favorable for determining the cellular localization of Cx45-IR puncta compared with the optical sections taken parallel to the retinal layers. Cx45-IR puncta were located over S100-labeled Müller cell somata in the middle of the INL in radial sections, as in the whole mounts. Pronounced labeling was also seen in the inner part of the INL in sections, but it was not possible to distinguish between the labeling of Müller cell processes and the labeling of neurons. In radial sections, which more accurately reflect the relative intensity of staining in the different retinal layers than do the confocal images through whole mounts, the Cx45 immunoreactivity in the ONL was much weaker than in the other layers. The monoclonal anti-Cx45 stained a single band at approximately 48 kDa in samples from retina (Fig. 1E). This antibody did not detect any bands on blots from brain (50 μg protein/lane). Peptide preabsorption eliminated staining of blots with this antibody. The AB1745 serum stained bands at approximately 48 kDa on blots of brain and retina, although this crude serum stained multiple additional bands on these blots (not shown).

**Cx26**

Cx26 has been demonstrated in ependymal cells (Dermietzel et al., 1989; Spray et al., 1991). Because the apical processes of Müller cells contact the subretinal space, a ventricular compartment, these cells have been characterized as ependymoglial cells (Reichenbach and Robinson, 1995). We therefore suspected that Müller cells might express Cx26. In initial experiments, we observed diffuse staining of astrocyte processes when using a polyclonal antibody directed against Cx26 (Zymed 71-0500), which has since been demonstrated to cross-react with Cx30 (Nagy et al., 1999). We failed to observe staining with the monoclonal anti-Cx26 (Zymed 13-8100) with any of the fixation protocols.
The purpose of this study was to identify the connexins in the rat retina that might form gap junctions between retinal glia. Immunostaining coupled with confocal microscopy allowed us to detect several connexins in the retina and to describe their laminar distribution.

**Cx43**

**Cx43 in astrocytes.** Using several different antibodies, we consistently observed that Cx43-IR puncta followed GFAP-IR astrocyte processes at the inner (vitreal) surface of the retina. These results imply that gap junctions formed by retinal astrocytes contain Cx43. The ubiquity of Cx43 in gap junctions formed by astrocytes in the brain (Dermietzel et al., 1989; Yamamoto et al., 1990a, 1991; Nagy et al., 1992, 1999; Dermietzel and Spray, 1993) and in culture (Dermietzel et al., 1991; Hofer and Dermietzel, 1998; Kunzelmann et al., 1999; Reuss et al., 2000) is well documented. Recent reports have confirmed the colocalization of Cx43 and GFAP immunoreactivity in the retinas of pigmented mice (Glodenagel et al., 2000; Söhl et al., 2000) and rabbits (Johansson et al., 1999). Cx43 immunoreactivity has also been detected at the inner limiting membrane (Jannsen-Bienhold et al., 1996) and in the nerve fiber layer of the retinas of albino rats (Janssen-Bienhold et al., 1998), which is consistent with an astrocytic localization. Cx43 mRNA has been detected in the RGC and nerve fiber layers of the mouse retina, which is consistent with localization to astrocytes (Söhl et al., 2000).

**Cx43 in Müller cells.** Two of the antibodies used in this study (the polyclonal Zymed 71-0700, lot 90647439, and the monoclonal Zymed 13-8300) labeled profiles associated with Müller cells. This result is consistent with findings in the rabbit retina, where Cx43 is symmetrically distributed on astrocyte–astrocyte, astrocyte–Müller cell, and Müller cell–Müller cell gap junctions (Johansson et al., 1999). However, Cx43 mRNA has not been reported to be present in the INL of the mouse retina, where the Müller cell somata are located (Söhl et al., 2000). Species differences might explain the presence of Cx43 protein in rat and rabbit Müller cells and the apparent absence of Cx43 mRNA in mouse Müller cells. Alternatively, Cx43 immunoreactivity in Müller cells might reflect the internalization by Müller cells of heterotypic astrocyte–Müller cell gap junctions, in which astrocytes contribute connexons formed by Cx43 and Müller cells contribute connexons formed by a different connexin. The internalization of the entire gap junction by one cell of a coupled pair has been demonstrated (Jordan et al., 2000). Although this possibility could explain our observations at the light level in the rat retina, it is not a likely explanation for the rabbit retina, in which Cx43 labels the Müller cell sides of gap junctions studied ultrastructurally (Johansson et al., 1999). Finally, in the absence of retinas from Cx43 null mice with which to test these antibodies, we cannot rule out the possibility that the antibodies recognize species other than Cx43 in the retina. In situ hybridization studies in rat and rabbit retinas are necessary to resolve this issue.

**Do the antibodies that stain astrocytes and Müller cells recognize differentially phosphorylated forms of Cx43?** Although two of the anti-Cx43 antibodies used in this study labeled Müller cells throughout their length, the other four anti-Cx43 antibodies exclusively labeled profiles associated with astrocyte processes at the vitreal surface. It is possible that some of this surface connexin immunoreactivity was on the Müller cell side of astrocyte–Müller cell gap junctions, which cannot be determined at the light microscopic level. Yet the question remains as to the differences in the epitopes recognized by polyclonal Zymed 71-0700, lot 90647439, and the monoclonal Zymed 13-8300, which clearly label Müller cells, and the epitopes recognized by the other antibodies. The monoclonal antibody Zymed 13-8300 has been reported to recognize Cx43 only when it is not phosphorylated at serine 368 (Nagy et al., 1997; Cruciani and Mikalsen, 1999). Our Western blots failed to demonstrate that the antibodies that differentially stained astrocytes and Müller cells recognized differentially phosphorylated isoforms of Cx43. All of the antibodies tested stained only single bands (as opposed to doublets or triplets) of ~43 kDa in samples from brain and retina, which suggests that Cx43 in the neural tissue underwent peri-mortem dephosphorylation in our preparations. Patterns of immunostaining for Cx43 have been shown to vary with the (patho-)physiological state of astrocytes (Rohllmann et al., 1993, 1994; Hossain et al., 1994a,c; ochalski et al., 1995; Nagy et al., 1996; Li et al., 1998; Li and Nagy, 2000), and these variations have been attributed to epitope masking (Ochalski et al., 1995). The differences in the staining patterns revealed by the different antibodies in this study imply that at least some of the Cx43 in Müller cells is in a state different from that of the Cx43 in astrocytes.

**Are there gap junctions between Müller cells?** The distribution of connexin immunoreactivity throughout the length of the Müller cells was somewhat surprising. We expected to observe connexin immunoreactivity associated with Müller cells in the inner retina, reflecting connexin trafficking to/from the Müller cell endfeet, where Müller cells are coupled to astrocytes (Robinson et al., 1993; Zahs and Newman, 1997). Cx43 immunoreactivity is present in the distal regions of Müller cells in fish (Giblin and Christensen, 1997; Ball and McReynolds, 1998) and amphibians (Ball and McReynolds, 1998), animals in which there are gap junctions between Müller cells (Uga and Smelser, 1973; Mobbs et al., 1988; Ball and McReynolds, 1998). It has been generally accepted that there are no gap junctions between mammalian Müller cells, but this view should be reconsidered. Although several ultrastructural
studies failed to demonstrate gap junctions associated with mammalian Müller cells (Uga and Smelser, 1973; Bussow, 1980; Holländer et al., 1991), the demonstration of tracer coupling between astrocytes and Müller cells (Robinson et al., 1993; Zahs and Newman, 1997) refutes the conclusion that Müller cells do not participate in any gap junctions. More recently, Johansson and colleagues (1999) have demonstrated Cx43-IR gap junctions between rabbit Müller cells. Functional coupling between mammalian Müller cells has yet to be conclusively demonstrated. Results of electrical coupling experiments in the rat retina are consistent with the presence of Müller cell-to-Müller cell gap junctions, but the interpretation of these data is complicated by the presence of intervening astrocytes (Ceelen et al., 2001). In the periphery of the rabbit retina, which contains Müller cells but no astrocytes, current injections into Müller cells evoke voltage changes in neighboring Müller cells (our unpublished observations), but we have been unable to demonstrate unequivocally that this current spread is via gap junctions.

We cannot eliminate the possibility that the Cx43 in Müller cells in the outer retina is present in gap junctions between Müller cells. Another possibility is that the Cx43 immunoreactivity is present in hemichannels. There is an increasing body of evidence that connexin hemichannels may serve as pathways for the release of signaling molecules from cells, including ATP (Cotrina et al., 1998) and NAD⁺ (Bruzzzone et al., 2000). ATP is released during the propagation of intercellular Ca²⁺ waves between retinal glia (Newman, 2001) and NAD⁺ acts extracellularly to evoke Ca²⁺ responses in Müller cells (Esguerra and Miller, 2002). It has recently been reported that current flow through Cx26 hemichannels in horizontal cells of the retina mediates feedback onto photoreceptors, providing the first example of a physiological role for hemichannels in a relatively intact tissue (Kamermans et al., 2001).

**Cx30**

Cx30 immunoreactivity was confined to the vitreal surface of the retina, where immunoreactive puncta colocalized with GFAP-IR astrocyte processes. Cx30 mRNA has not been detected in the mouse retina using reverse transcriptase-polymerase chain reaction (RT-PCR; Güldenagel et al., 2000). The absence of Cx30 in the mouse retina may reflect species differences between the rat and the mouse.

Cx30 has been demonstrated in astrocytes throughout the brain and spinal cord; immunoreactivity is pronounced between astrocyte endfeet abutting blood vessels (Kunzelmann et al., 1999; Nagy et al., 1999). These results are consistent with our finding that Cx30-IR puncta colocalize with GFAP-IR astrocyte processes and with our impression that these puncta are frequently located where the astrocyte processes contact the superficial retinal blood vessels. In the brain, Cx30 immunoreactivity is much greater in gray matter than in white matter tracts. Retinal astrocytes are immigrants from the optic nerve (Watanabe and Raff, 1988), yet they display robust Cx30 immunoreactivity. These findings suggest that the set of connexins expressed by astrocytes is at least partly a function of the local environment of the cells and does not depend only on cell lineage.

Cx30 immunoreactivity in brain astrocytes frequently colocalizes with Cx43 immunoreactivity, although the onset of Cx30 expression occurs after the onset of expression of Cx43 (Dahl et al., 1996; Kunzelmann et al., 1999; Nagy et al., 1999). It has been suggested that gap junctions composed of Cx30 and Cx43 may have different permeabilities to lactate or other glucose metabolites (Nagy et al., 1999) and that gap junctions composed of Cx30 may have a special role in K⁺ spatial buffering (Kunzelmann et al., 1999).

**Cx45**

Immunoreactive puncta were observed throughout the depth of the retina with three different antibodies directed against Cx45. Cx45-IR profiles were found primarily over glutamine synthetase- or S100-IR Müller cell processes. In addition, Cx45-IR puncta were found over S100-labeled astrocyte processes, which were distinguishable from Müller cell processes by their shape and location. Cx45 mRNA has been detected in the mouse retina by RT-PCR, and a lacZ reporter gene substituted for the open reading frame of Cx45 was expressed in RGCs and somata in the INL (Güldenagel et al., 2000). Using frozen sections of the mouse retina, Güldenagel et al. also found Cx45 immunoreactivity in the inner retina as well as in the outer plexiform layer, and they concluded that Cx45 is present in amacrine cells and possibly some horizontal cells. Our observations are consistent with the expression of Cx45 by some amacrine cells. In confocal images collected parallel to the retinal layers, it is apparent that the pronounced Cx45 immunoreactivity surrounding horizontal cells is localized to the Müller cell processes surrounding these neurons (Fig. 5).

Outside of the retina, Cx45 is present in oligodendrocytes (Dermietzel et al., 1997; Kunzelmann et al., 1997). Cx45 mRNA and protein have also been detected in cultured astrocytes (Dermietzel et al., 2000b).

**Potential composition of gap junctions between retinal glia**

In the current study, immunoreactivity for Cx30, Cx43, and Cx45 colocalized with astrocyte markers, while immunoreactivity for Cx34 and Cx45 colocalized with Müller...
Figure 4
Fig. 4. Cx45 immunoreactivity colocalizes with retinal glial cells. Paraformaldehyde-fixed retina doubly immunostained using Chemicon MAB3101 directed against Cx45 (green) and a polyclonal antibody directed against S100 (red). A–F: Each panel represents the superimposition of six confocal images collected at 0.2 μm intervals in a plane parallel to the retinal layers. Anti-S100 stains astrocytes at the vitreal surface (A) and Müller cells throughout the depth of the retina (B–F). Arrows in C mark a linear array of Cx45-IR puncta, which may represent staining of a neuronal process. Asterisks in E mark the somata of two presumed horizontal cells, which are surrounded by heavily Cx45-IR Müller cell (S100-IR) processes. G–K: Retinal sections. Retinal section (transmitted light image shown in G) doubly immunostained with MAB3101 (shown in H and in green in pseudocolor overlay in J) and S100 (shown in I and in red in pseudocolor overlay in J). K: Peptide preabsorption eliminates staining with antibody MAB3101: retinal section doubly immunostained with anti-S100 (red) and with monoclonal antibody MAB3101 that had been incubated with peptide antigen (green). Scale bar in F = 10 μm for A–F; bar in K = 15 μm for G–K.

Fig. 5. Cx45 immunoreactivity in Müller cell processes that surround horizontal cells. The field shown in Figure 4E is enlarged, and the staining with the anti-Cx45 (A) and anti-S100 (B) antibodies are shown separately. Boxes are drawn around two corresponding regions in each image to facilitate comparison of the locations of the connexin staining and the S100-immunoreactive Müller cell processes. Scale bar = 5 μm.

cell markers. Several patterns of connexin expression by retinal glia could result in this pattern of immunoreactivity observed at the light microscopic level: 1) Retinal astrocytes express Cx30, Cx43, and/or Cx45, while Müller cells express Cx43 and/or Cx45. 2) One or more of the connexins that colocalize with astrocyte processes (-30, -43, -45) are actually localized to the Müller cell side of astrocyte–Müller cell gap junctions. 3) Cx43 and/or Cx45 are expressed exclusively in astrocytes in the retina, and the immunoreactivity for these connexins observed in Müller cells is due to internalization by Müller cells of heterotypic gap junctions (with Cx43 or Cx45 on the astrocyte side of the gap junction). This latter possibility seems unlikely, in that connexin immunoreactivity is observed throughout the length of the Müller cell. If this immunoreactivity is indeed present in heterotypic gap junctions that are being degraded, we would expect to have seen this immunoreactivity primarily in the inner retina, in the proximal stalks of the Müller cells. In situ hybridization studies will be necessary to localize the mRNAs for these connexins to specific cell types in the rat retina. However, based on the results presented in this study, as well as results from studies of the expression and localization of connexins in brain glia, we propose that retinal astrocytes express Cx30 and Cx43 and possibly Cx45, while Müller cells express Cx43 and Cx45. In addition, Cx50 has been localized to Müller cells and astrocytes in the retinas of albino rats (Schutte et al., 1998).

The pattern of immunoreactivity that we observe in the retina is consistent with several possible astrocyte–Müller cell gap junctions: Cx43/Cx43, Cx45/Cx45, Cx43/Cx45, and Cx45/Cx43. By including the results of Schutte et al. (1998), this list can be expanded to include the possibility of homotypic astrocyte–Müller cell gap junctions composed of Cx50. The possibility that there are heteromeric connexons in one or both cell types increases the number of potential types of gap junctions that could form between these cells.

The asymmetry of the coupling between retinal astrocytes and Müller cells (Robinson et al., 1993; Zahs and Newman, 1997) is consistent with the presence of heterotypic gap junctions between these cells (Flagg-Newton and Loewenstein, 1980). The results of ultrastructural immunohistochemical studies of brain glial cells have led several authors to suggest that astrocyte–oligodendrocyte gap junctions may be composed of Cx43/Cx45 and/or Cx30/Cx32 (Dermietzel et al., 1997; Kunzelmann et al., 1997; Li et al., 1997; Nagy et al., 1999). In addition to the expression of Cx45, Müller cells share other properties with oligodendrocytes, including the expression of carbonic anhydrase (Linser et al., 1984) and cellular retinaldehyde-binding protein (Saari et al., 1997) and a morphological relationship with retinal ganglion cell axons (Holländer et al., 1991). Considering the functional properties shared by Müller cells and oligodendrocytes, we propose that...
The biophysical and electrophysiological properties of gap junctions were shown to be modulated by the presence of Cx43/Cx45 also make them likely candidates for the gap junctions between retinal astrocytes and Müller cells. Under our experimental conditions, astrocytes are ~10 mV depolarized relative to Müller cells (Zahs and Newman, 1997). The voltage gradient that would exist across astrocyte–Müller cell gap junctions could be significant if Cx45 contributes to these channels. At least in the Xenopus expression system, gap junctions formed by Cx45 are highly voltage-dependent: The steady-state junctional conductance is reduced to 50% of maximal levels at transjunctional voltages of ±14 mV (Steiner and Ebihara, 1996) compared with ~55 mV for Cx43 (White et al., 1994). Furthermore, heterotopic Cx43/Cx45 channels display asymmetric voltage dependences, such that hyperpolarization of the Cx43 side of the junction causes a slow inactivation of junctional current, while depolarization of the Cx43 side of the junction causes an increase in junctional current (Steiner and Ebihara, 1996). Under the conditions of our experiments, if the astrocyte–Müller cell gap junctions are indeed formed by Cx43/Cx45, we expect a significant fraction of these channels to be closed. Conditions that lead to a reduction of the voltage gradient between astrocytes and Müller cells are expected to increase the conductance of these gap junctions.

**Regulation of coupling**

The existence of multiple connexins within retinal glia could endow these cells with a set of diverse gap junction channels that vary in their selective permeabilities and modes of regulation. Gap junctions between astrocytes are permeable to the tracer Lucifer yellow, but gap junctions between astrocytes and Müller cells are not (Robinson et al., 1993; Zahs and Newman, 1997). Channels formed by different connexins vary in their permeabilities to biological signaling molecules, including inositol trisphosphate (Niessen et al., 2000) and nucleotides (Bevans et al., 1998; Nicholson et al., 2000). It remains to be determined how gap junctions between retinal glia differ in their permeabilities to biologically relevant molecules.

There is also little information on the regulation of gap-junctional coupling between retinal glia. Astrocyte–astrocyte gap junctions and astrocyte–Müller cell gap junctions differ in their sensitivities to pharmacological uncoupling agents (Zahs and Newman, 1997), but there are no data regarding the effects of neuromodulators or physiological conditions on coupling between retinal glia. There is extensive gap-junctional coupling between neurons in the mammalian retina, and neuronal coupling can be modulated by light exposure (Bloomfield et al., 1997; Xin and Bloomfield, 1999) and by neuromodulators (DeVries and Schwartz, 1989; Miyachi et al., 1990; Hampson et al., 1992; Mills and Massey, 1995; Weiler et al., 1999; He et al., 2000). Factors derived from the vasculature, including nitric oxide (DeVries and Schwartz, 1989; Miyachi et al., 1990; Bolanos and Medina, 1996; Yang and Hatton, 1999) and endothelin-1 (Tabernero et al., 1996), have been shown to modulate junctional coupling between cultured astrocytes, and there is circumstantial evidence that a vascular factor might differentially influence astrocyte–astrocyte and astrocyte–Müller cell coupling (Zahs and Wu, 2001).

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**LITERATURE CITED**


CONNECXINS IN RAT RETINAL GLIA


