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Differential Expression of Connexins During Histogenesis of the Chick Retina

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Abstract

Gap junction (GJ) channels couple adjacent cells, allowing transfer of second messengers, ions, and molecules up to 1 kDa. These channels are composed by a multigene family of integral membrane proteins called connexins (Cx). In the retina, besides being essential circuit element in the visual processing, GJ channels also play important roles during its development. Herein, we analyzed Cx43, Cx45, Cx50, and Cx56 expression during chick retinal histogenesis. Cx exhibited distinct expression profiles during retinal development, except for Cx56, whose expression was not detected. Cx43 immunolabeling was observed at early development, in the transition of ventricular zone and pigmented epithelium. Later, Cx43 was seen in the outer plexiform and ganglion cell layers, and afterwards also in the inner plexiform layer. We observed remarkable changes in the phosphorylation status of this protein, which indicated modifications in functional properties of this Cx during retinal histogenesis. By contrast, Cx45 showed stable gene expression levels throughout development and ubiquitous immunoreactivity in progenitor cells. From later embryonic development, Cx45 was mainly observed in the inner retina, and it was expressed by glial cells and neurons. In turn, Cx50 was virtually absent in the chick retina at initial embryonic phases. Combination of PCR, immunohistochemistry and Western blot indicated that this Cx was present in differentiated cells, arising in parallel with the formation of the visual circuitry. Characterization of Cx expression in the developing chick retina indicated particular roles for these proteins and revealed similarities and differences when compared to other species.

Keywords

development; gap junction; connexin; retina; gene expression; histogenesis

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Additional Supporting Information may be found in the online version of this article.

INTRODUCTION

Gap junction (GJ) channels couple adjacent cells, allowing the passage of ions and small molecules up to 1 kDa. These channels are formed by two hexameric arrays of connexins (Cx), which constitute a multigene family of integral membrane proteins (Willecke et al., 2002; Sohl et al., 2004). The association of six Cxs forms a hemichannel (connexon), which has been proposed to operate as an independent functional element (for review, see Evans et al., 2006). The docking of two hemichannels in apposed cell membranes makes GJ channels, where gating and permeation properties differ depending on the combination of Cxs (Veenstra, 1996).

In the retina, most of the knowledge regarding Cx expression has arisen from studies in mammalian nocturnal species, in which visual system is adapted for scotopic vision (Condorelli et al., 1998; Guldenagel et al., 2000; Kihara et al., 2006). In the mouse retina, Cx36 and Cx45 were identified as mainly neuronal Cxs participating in the rod-mediated circuitry (Deans et al., 2002; Maxeiner, 2005; Kihara et al., 2008), whereas Cx57 is apparently restricted to horizontal cells (Hombach et al., 2004). Other Cxs such as Cx37 and Cx43 seemed to be expressed exclusively by endothelial (Guldenagel et al., 2000) and glial cells (Zahs et al., 2003), respectively. Interestingly, in the rabbit retina, Cx50 was reported in horizontal cells (O'Brien et al., 2006) and glial cells (Schütte et al., 1998).

In addition to circuitry-related roles attributed to Cxs in the mature retina, Cx-mediated communication also participates in several processes during retinal development. For instance, GJ channels are involved in the establishment of regulatory compartments during programmed cell death, which helps to establish the distribution of the various cell classes into distinct layers of the retina (Cusato et al., 2003). Also important is the spontaneous activity that propagates as waves of action potentials in retinal cells, driving rhythmic increases in intracellular calcium. These retinal waves are mediated by a combination of chemical and electrical synaptic transmission (Wong, 1999). Finally, it was proposed that ATP released from pigmented epithelium to the developing retina passes through Cx hemichannels located nearby the neuroblastic ventricular zone (Pearson et al., 2005), thereby controlling cell proliferation.

Although the mentioned processes are essential in retinal morphogenesis and require Cx-mediated communication, the spatiotemporal expression of these genes remains largely unknown. In this context, the aim of this study was to investigate the expression of Cx43, Cx45, Cx50 (also known as chCx45.6), and Cx56 during the development of the chick retina. The use of proper antibodies may be critical to the correct protein detection. Most of the available Cx antibodies were raised against epitope of human/rat/mouse Cxs. However, detailed analysis of Cx amino acid sequence revealed that most of these commercially available antibodies do not recognize chick Cxs. Herein, we have used solely antibodies that are specifically raised against chick Cx (Cx50 and Cx56) or that recognizes epitopes that share 100% homology with the respective Cx in the chick (Cx43 and Cx45).

METHODS

Animal Procedures

Experiments were carried out with *Gallus gallus*, ranging from embryonic day 4.5 (E4.5) to 15 days after hatching (E4.5, E7, E12, E15, E18, and P15). Animals were killed for different methodologies with an overdose of ketamine (30 mg/100 g of body weight, i.m., Parke-Davis, Ann Arbor, MI) and xylazine (2 mg/100 g, i.m., West Haven, CT). These experiments were conducted in accordance with NIH and Institute of Biomedical Sciences/University of São Paulo guidelines.

RNA Isolation, cDNA Synthesis, and Real-Time PCR

Retinas were directly homogenized in 1 mL TRIzol reagent (Invitrogen, Carlsbad, CA), and total RNA was extracted following the manufacturer's suggested protocol. Briefly, following two chloroform extraction steps, RNA was precipitated with isopropanol and the pellet washed twice in 70% ethanol. After air-drying, RNA was resuspended in DEPC-treated water, and the concentration of each sample was obtained from A_{260} measurements. Residual DNA was removed using DNase I (Amersham, Piscataway, NJ) following the manufacturer's protocol. For each 20 μ L of the reverse transcription reaction, 4 μ g total RNA was mixed with 1 μ L oligodT primer (0.5 μ g; Invitrogen) and incubated for 10 min at 65°C. After cooling on ice, the solution was mixed five times with 4 μ L first strand buffer, 2 μ L of 0.1 MDTT, 1 μ L of dATP, dTTP, dCTP and dGTP (each 10 mM), and 1 μ L Superscript III reverse transcriptase (200 U; Invitrogen) and incubated for 60 min at 50°C. Reaction was inactivated by heating at 70°C for 15 min. Quantitative analysis of Cx gene expression was carried out using Rotor-Gene 6000 Real-Time Rotary Analyzer (Corbett Robotics, San Francisco) with specific primers for different Cxs (Table 1). cDNA abundance for GAPDH was also determined as internal control. All PCR assays were performed as follows: after initial activation at 50°C for 2 min and 95°C for 10 min, cycling conditions were 95°C, 10 s and 60°C, 1 min. Dissociation curves of PCR products were obtained by heating samples from 60 to 95°C, in order to evaluate primer specificity.

PCR Statistical Analysis

Relative quantification of target gene expression was evaluated using the comparative CT method as previously described in detail (Medhurst et al., 2000). The CT value was determined by subtracting the target CT of each sample from its respective GAPDH CT value, used as internal control. Calculation of CT involves using the CT mean of control group (in the present study, P15) as an arbitrary constant to subtract from all other experimental CT mean values (developmental ages). Fold changes in gene expression of the target gene are equivalent to 2^{-CT} . Values were entered into a one-way analysis of variance (ANOVA), followed by pairwise comparisons (Tukey's HSD test), with significance level set at 5%.

Immunohistochemistry

Previous studies have mentioned that paraformaldehyde (PFA) caused nonspecific labeling when some Cx antibodies were used (Meier et al., 2002). Thus, we tested two different

fixation protocols, using PFA and ethanol (ETOH). Eyes were dissected out and the retinas were fixed for 30 min in 1% PFA in phosphate buffer 0.1 M pH 7.3 (PB), and cryoprotected in 30% sucrose solution for at least 24 h at 4°C. After embedding in optimum cutting temperature mounting medium (O.C.T., Sakura Finetek, Torrance, CA), they were cut transversally (12 μ m) on a cryostat. Retinal sections were blocked for 30 min in a solution containing 10% normal goat serum, 1% bovine serum albumin (BSA) and 0.5% Triton-X 100 in PB. Alternatively, eyes were removed, rapidly embedded in OCT, and frozen in 8% methylcyclohexan in 2-methylbutan (v/v; -60°C) as previously described (Degen et al., 2004). Sections were fixed in cold ethanol (-20°C) for 10 min, rinsed in PBS, and preincubated in blocking buffer (10% normal goat serum and 0.5% Triton X-100 in PBS) for 30 min. Immunoreactivity patterns were very similar with both fixatives for Cx antibodies, as we previously reported (Kihara et al., 2006). Because preservation and immunostaining of the tissue were better with PFA, we decided to use this fixative.

To identify Cx43, we used a polyclonal antibody against amino acids 251–270 of rat Cx43 (no. 71-0700, Zymed/Invitrogen, 1:200). This amino acid sequence shares 100% homology with chick Cx43. This antibody has been used in rodent retinal studies (Zahs et al., 2003), and its specificity was confirmed by reduced labeling in retinas of Cx43-deficient mice (Kihara et al., 2006). In the present study, preadsorption controls using immunogenic peptide indicated the specificity of the antibody. In order to investigate Cx45 protein distribution, we used an antibody donated by Dr. Thomas Steinberg and Dr. Michael Koval (1:200–1:500). This antibody was raised against amino acids 285–298 of dog Cx45 (Kanter et al., 1992; Steinberg et al., 1994), which shares 100% homology with chick Cx45. In this study, we observed that this antibody recognizes a doublet band at the expected molecular weight in Western blots, suggesting its specificity. Furthermore, this antibody was previously employed in neuronal studies (Chang et al., 1999) and also shown to recognize chick Cx45 transfected into rat osteoblastic cells (Koval et al., 1995). To determine spatial distribution Cx50, we used a purified antibody specifically raised against C-terminal portion of Cx45.6 (1:50–1:200), the chick ortholog of mammalian Cx50. This anti-Cx50 was extensively characterized in chick lens studies (Jiang et al., 1994; Yin et al., 2000). In the present study, elimination of staining in preadsorption controls suggested the specificity of this antibody. To investigate Cx56, we used an affinity-purified antibody raised against C-terminal portion of chick Cx56 (1:50–1:200). This antibody was previously characterized in chick lens studies (Jiang et al., 1994). Herein, positive controls were performed in Western blots and sections of chick lens to attest immunoreactivity of the antibody.

In order to determine the expression of Cx in specific retinal cell types, double-labeling experiments were carried out using purified antibodies raised against neurofilaments (PAN), glial fibrillary acidic protein (GFAP), and calretinin (CR). The anti-PAN (no. 18-0171, Zymed/Invitrogen, 1:200) is a cocktail of three mouse monoclonal antibodies which reacts with all three major neurofilament polypeptides designated as low (68 kDa), medium (160 kDa), and high (200 kDa), based on their molecular weight. Neurofilament immunolabeling has been used as a marker for ganglion cell processes in the chick retina (Brocco and Panzetta, 2002; Shin et al., 2003). A mouse monoclonal anti-GFAP (no. G3893, Sigma-Aldrich, St. Louis, MO, 1:2000) was used to identify Müller glial cells, as described in other studies (Torelli et al., 1989). Finally, we also used a goat polyclonal anti-CR (no. AB1550,

Chemicon, 1:200) to label amacrine cells. CR expression by amacrine cells was previously characterized in the chick retina (for review, see Drenhaus et al., 2004).

After several washes, retinal sections were incubated with goat antisera against rabbit or mouse IgG tagged to Alexa TM 488 (Molecular Probes, Eugene, Oregon, 1:250–1:500) diluted in PB-containing 0.5% Triton X-100 for 2 h at room temperature. For double-labeling experiments, secondary antibodies conjugated to fluorescein isothiocyanate (Jackson Labs, West Grove, PA, 1:250–1:500), to tetramethyl rhodamine isothiocyanate (Jackson Labs, 1:250–1:500), or to indodicarbocyanine (Cy5, Jackson Labs, 1:250–1:500) were used. Controls for the experiments consisted of the omission of primary antibodies; no staining was observed in these cases. In order to better visualize retinal layers, we performed counterstaining using propidium iodide. After washing, the tissue was mounted using Vecta Shield (Vector Labs, Burlingame, CA), and analyzed in a PCM2000 confocal microscope (Nikon, Yokohama, Kanagawa, Japan). Figures were mounted with Photoshop CS2 (Adobe, San Jose, CA). Manipulation of the images was restricted to threshold and brightness adjustments to the whole image.

Western Blotting

Retinas were rapidly dissected and homogenized in 25 mM Tris buffer containing a cocktail of protease inhibitors (Calbiochem, La Jolla, CA). Homogenates were centrifuged for 5 min at 2000g (4°C) to remove insoluble material. The supernatant was centrifuged at 100,000g (4°C) for 30 min to obtain the crude membrane fraction and the protein concentration was determined by the Bradford method. BSA was used as the standard. Proteins in the membrane preparations were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (4–20% gradient gel) and transferred to nitrocellulose membranes. Blots were incubated with 5% powdered milk in PBS with 0.2% Tween-20 for 1 h at room temperature to block nonspecific binding of the antibodies. In addition to the antibodies previously described, we also used a monoclonal antibody raised against amino acids 360–376 of rat Cx43 (no. 13-8300, Invitrogen), as described in previous studies (Li et al., 1998). This anti-Cx43 recognizes Cx43 only when the serine at residue 368 is unphosphorylated, since this serine is located within the epitope recognized by this antibody. After they were rinsed in PBS, blots were incubated overnight at 4°C with primary antibodies raised against Cx43 (1:1000 and 1:500, polyclonal and monoclonal, respectively), Cx45 (1:1000), Cx50 (1:1000), and β -actin (no. A5316, Sigma-Aldrich, 1:1000) diluted in PBS/0.2% Tween-20/1% powdered milk. After the primary antibody incubation, blots were rinsed in PBS/0.2% Tween-20/5% powdered milk and incubated with goat anti-rabbit or -mouse peroxidase (ECL™ kit; Amersham, Buckinghamshire, England) for 2 h at room temperature. Detection of labeled proteins was achieved by using the enhanced chemiluminescent system (ECL kit; Amersham).

Measurements of optical densities were performed using Scion Image software (Scion Corporation, Frederick, MD). Optical densities of the bands from three independent experiments were normalized by the respective value obtained for mature retina (P15). Data entered into a oneway ANOVA followed by pairwise comparisons in Tukey's HSD test.

RESULTS

Detection of Cx45 and Cx50 in the Chick Retina

Previous studies have not been able to detect major Cx in the chick retina, such as Cx36 and Cx45 (Becker et al., 2002). In the present study, by using a more precise and sensitive technique such as realtime PCR (Schmittgen et al., 2000; Kihara et al., 2005), we were able to first detect Cx45 transcripts in the chick retina (see Fig. 1). By using primers specifically designed for chick Cx45 we generated amplification plots from cDNA serial dilutions [Fig. 1(A)]. Dissociation curves of these PCR products were obtained by heating samples from 60 to 95°C. The single peak observed matched to theoretical melting temperature calculated previously, indicating specificity of the primers [Fig. 1(B)]. Linear regression analysis of amplification plots revealed high correlation, confirming amplification linearity [Fig. 1(C)]. With the same procedures, we also detected Cx50, but failed to detect Cx56 mRNA in chick retinas [Fig. 1(C), arrow], although positive controls performed in chick lens indicated primer integrity. Accordingly, we were not able to immunodetect chick Cx56 in Western blots and in retinal sections by using purified antibodies raised against this Cx (data not shown).

Cx Gene Expression Is Specifically Regulated During Chick Retinal Development

By using specific primers designed for chick Cxs, we also determined that these genes (Cx43, Cx45, and Cx50) have distinct expression profiles during chick retinal histogenesis. Our quantitative PCR results indicated that Cx43 gene is highly expressed in E4.5 (67-fold, $p < 0.01$) and E7 retinas (64-fold, $p < 0.01$) when compared to P15 [Fig. 2(A)]. On the other hand, Cx45 transcripts showed constant steady-state levels during the entire development of the retina [Fig. 2(B)]. Finally, real-time PCR experiments revealed that Cx50 gene is poorly expressed in retinas from 4.5- (23%, $p < 0.01$) and 7- (35%, $p < 0.01$)-day-old embryos when compared to P15 [Fig. 2(C)]. In these PCR experiments, GAPDH gene expression was used as internal control [Fig. 2(D)].

Distribution Pattern of Cxs in the Embryonic and Adult Retina

By using purified antibodies raised against chick Cxs, we analyzed the distribution pattern of Cx43, Cx45, and Cx50 during chick retinal development. Retinal neurogenesis follows an evolutionary conserved order, starting with the differentiation of retinal ganglion cells and horizontal cells, followed in overlapping phases by cones, amacrine cells, rods, bipolar cells, and Müller glial cells (Carter-Dawson and LaVail, 1979; Young, 1985; Prada et al., 1991). With the exception of rods, which are virtually absent, all cell types differentiate in the period comprising E2–E3 to E10–E12 in the chick retina (Prada et al., 1991).

Spatial–Temporal Expression of Cx43 in the Retina

At E4.5, we found an intense labeling in the outer part of the neuroblastic layer, a region known as ventricular zone. As shown in Figure 3, the transition of the pigmented epithelium and the neuroblastic layer is quite subtle, although nuclei counterstained with propidium iodide (red) permitted this distinction. At E7, Cx43 immunolabeling formed a thin line between the neuroblastic layer and the pigmented epithelium. In fact, at these early ages

(E4.5 and E7), messy, disordered puncta were seen between the cells of pigmented epithelium. From E12 to mature retina, the labeling in the pigmented epithelium depicted the typical hexagonal format of these cells (not shown at E18 and P15). In the E12 retinas, we observed the punctate labeling in the outer plexiform layer. Although not as evident, puncta in the inner part of the inner nuclear layer and in the ganglion cell layer were seen. At E15, Cx43 staining was observed in the ganglion cell layer and in the outer plexiform layer. Also, a few puncta were seen in the outer margin of the outer nuclear layer and in the inner part of the inner nuclear layer. At E18 and P15, we observed labeling forming lines in both outer and inner plexiform layers, in addition to puncta within the ganglion cell layer (see Fig. 3).

In the adult retina, Cx43 labeling was seen in the ganglion cell layer, suggesting that this protein is expressed by ganglion cells. In order to determine whether this Cx is expressed by ganglion cell processes in the adult retina, double-labeling experiments were carried out using anti-PAN neurofilament, a marker for neuronal processes (see Fig. 4). Indeed, we observed colocalization of Cx43 and neurofilament in the inner part of the ganglion cell layer, indicating that Cx43 is present in processes of ganglion cells. In addition, we also observed puncta surrounding presumptive ganglion cell perykaria which did not colocalized with neurofilament.

Spatial–Temporal Expression of Cx45 in the Retina

We observed a ubiquitous Cx45 distribution throughout chick retinal histogenesis, especially at early ages. At E4.5, the characteristic Cx punctate immunolabeling was seen in the entire retina (see Fig. 5). At E7, Cx45 staining was observed in the neuroblastic layer, especially at the inner part. In this region, the typical discrete labeling was seen, although these puncta were also present forming aggregates. In E12 retinas, when all retinal layers were depicted, Cx45 labeling was seen mainly in the inner plexiform layer, in the ganglion cell layer and in the nerve fiber layer. At E15, a similar distribution was observed, although some immunoreactivity was occasionally observed in the inner part of the inner nuclear layer. The spatial pattern described for E15 did not significantly change in vertical sections of E18 and P15 retinas (see Fig. 5).

In previous studies carried out in mouse retina, we were able to determine that Cx45 is expressed in Muller glial cells, in addition to neurons (Kihara et al., 2006). In order to investigate the putative glial expression of this Cx in the adult chick retina, we performed double-labeling experiments using GFAP, a marker for glial cells. In the chick retina anti-GFAP stains vertical processes of Muller glial cells especially in the inner retina, as similarly described for anti-vimentin (Santos-Bredariol et al., 2006), but depending on the concentration immunolabeling also depicts processes running vertically in the outer retina [Fig. 6(A)]. High magnification of double-labeling images permitted identification of Cx45 [Fig. 6(B,D,F)] and GFAP [Fig. 6(C,E,G)] colocalization. However, it was also possible to identify Cx45 punctate labeling that clearly did not colocalize with GFAP.

Spatial–Temporal Expression of Cx50 in the Retina

We were not able to detect Cx50 immunolabeling in vertical sections of retinas from 4.5- and 7-day-old embryos (see Fig. 7). At E12, Cx50 labeling in the retina was seen as a faint

sparse punctate located mainly in the outer and inner plexiform layers, and also in the transition of the latter and the ganglion cell layer (see Fig. 7). In E15 retinas, the characteristic Cx labeling was seen in both outer and inner plexiform layers, and also in the ganglion cell layer and in the inner half of the inner nuclear layer. A similar pattern was observed in vertical sections sampled from E18 and P15 central retinas, although punctate density seemed to increase over time, especially in the inner plexiform layer (see Fig. 7).

In the adult retina, Cx50 labeling was seen within the inner plexiform layer, where the expression could be assigned to several cell types, including ganglion cells and Muller glial cells, but most likely bipolar and amacrine cells, heralded by the labeling observed in the inner part of the inner nuclear layer. To examine this hypothesis, we performed double-labeling experiments using CR, a marker for amacrine cells in the chick retina (see Fig. 8). In the chick retina, anti-CR depicts horizontal laminae in the inner plexiform layer, corresponding to processes from amacrine cells located in the inner nuclear layer and cells in the ganglion cell layer. Double-labeling sections analyzed under confocal microscopy revealed that Cx50 colocalizes with CR, indicating that Cx50 is expressed by amacrine cells. Interestingly, we could also observe a few puncta which did not colocalize with CR labeling.

Cx Protein Levels Are Specifically Regulated During Chick Retinal Development

Western blots experiments were performed to determine Cx protein levels during histogenesis of the chick retina [Fig. 9(A)]. Densitometric analysis of bands from three independent experiments using the rabbit anti-Cx43, an antibody whose reactivity with Cx43 is independent of the phosphorylation status, revealed that this protein is highly expressed at E4.5 (255%, $p < 0.01$) when compared to P15, with no significant differences in the other developmental ages [Fig. 9(B)]. Cx43 phosphorylation results in distinct Cx43 forms migrating ranging around 41 and 43 kDa with greater degrees of phosphorylation giving rise to bands located at higher molecular weights (Musil et al., 1990; Laird et al., 1991). We observed phosphorylated forms of Cx43 throughout the embryonic development and in the mature retina, as well. The mouse anti-Cx43, otherwise, recognizes Cx43 only when the serine at residue 368 is unphosphorylated. Previous studies indicated that this antibody also recognizes slower migrating bands in addition to the band located at 41 kDa (Nagy and Li, 2000). These slower bands represent phosphorylated forms of Cx43, since phosphorylation may occur in sites other than serine 368. Using this mouse anti-Cx43, we observed that the expression of this particular subset of Cx43 in retinas of E4.5 (35%, $p < 0.01$), E7 (25%, $p < 0.01$) and E15 (29%, $p < 0.01$) embryos was very poor when compared to the mature retina [Fig. 9(C)]. Moreover, we observed the presence of slower bands in the homogenates from P15 retinas. In turn, quantification of Cx45 bands indicated that optical density did not vary through studied ages, although we observed single bands at E4.5–E15, and doublet bands at P15 [Fig. 9(D)]. Finally, Cx50 was shown to be virtually absent in retinas from E4.5 (4%, $p < 0.01$) and E7 (9%, $p < 0.01$) embryos, and poorly expressed in E15 (29%, $p < 0.01$) when compared to the mature retina [Fig. 9(E)]. In these Western blot experiments, β -actin was used as an internal control.

DISCUSSION

Cx43: Biphasic Expression in the Chick Retina

Cx43 was the first cloned and sequenced Cx, and its ubiquitous expression has been extensively characterized in different vertebrate tissues (for review, see Cruciani and Mikalsen, 2007). In the central nervous system, Cx43 was mainly described in glial cells (Theis et al., 2005), despite a few reports of expression of Cx43 by neurons in specific regions (for review, see Rozental et al., 2000). In accordance, Cx43 has been described in glial cells in the adult rodent retina (Guldenagel et al., 2000; Zahs et al., 2003; Kihara et al., 2006). Interestingly, in the embryonic chick retina, Cx43 expression was determined in the transition of the retinal pigmented epithelium (RPE) and the neural retina (Pearson et al., 2004).

In the present study, by following the expression of Cx43 during chick retinal histogenesis using quantitative methods, we verified the high expression of Cx43 in the early embryonic development. In addition, we observed important, complex changes in the phosphorylation state of Cx43 during retinogenesis. Using an antibody whose reactivity with Cx43 is independent of the phosphorylation status, we determined relative high protein levels at initial developmental stages and the presence of phosphorylated forms of this protein during all embryonic stages and in the mature retina, as well. Additionally, “filtering” Cx43 expression by using an antibody that recognizes the protein only when its epitope is dephosphorylated (therefore, a subset of the total Cx43), we observed phosphorylation at residues other than serine 368 particularly in the mature retina. Taking together, these results revealed a complex, intricate pos-translational control of Cx43. Indeed, several lines of evidence indicated that Cx43 phosphorylation status correlates to GJ permeability (Musil et al., 1990; Lampe and Lau, 2000), although pos-translational regulation of Cx43-mediated communication encompasses stimulus/tissue specificities (Li et al., 1998).

Confirming previous reports, we observed Cx43 in the transition of RPE and retina at E4.5–E7, where hemichannels formed by this Cx might allow the passage of ATP and other molecules (Pearson et al., 2004). Interestingly, from later embryonic development through mature retina, we determined a gradual, increased expression of Cx43 in both inner plexiform layer and ganglion cell layer. Our doublelabeling experiments revealed that this expression highly colocalized with neurofilament in the inner retina, indicating that Cx43 expression at this location was probably neuronal. Taking into account of its spatial pattern and also double-labeling data, this particular expression of Cx43 could be assigned to ganglion cells. In accordance, previous studies characterized dye coupling in E14 retinal ganglion cells, when/where Cx32 and Cx43 were shown to be expressed (Becker et al., 2002). In summary, we observed unequivocal, clear changes of Cx43 expression along retinal development, which is probably related to specific, differential roles of the communication mediated by this Cx in the developing and mature retina.

Cx45 Is Highly Expressed by Progenitor Cells

In contrast to Cx43, Cx45 expression had not been characterized in the chick retina so far. In this study, we were able to determine high gene expression levels by quantitative PCR as

well as constant, steady-state protein levels by Western blot. Furthermore, we could determine a ubiquitous distribution of Cx45 during early histogenesis of the retina. Indeed, during initial embryonic development (E4.5), Cx45 labeling was observed in the entire neuroblastic layer, when/where most cells undergo proliferation and/or differentiation. Following the maturation of the retina, Cx45 expression was progressively confined to inner retinal layers, which suggested its putative expression by specific cell populations. Interestingly, doublelabeling experiments with GFAP simultaneously indicated that Cx45 is expressed by glial cells, and by exclusion, in neurons. In spite of this finding, what seems to be consensual is that Cx45 is expressed by retinal neurons (Guldenagel et al., 2000; Kihara et al., 2006). Nonetheless, since Cx45 in the retina was shown to be essential in the rod pathway (Maxeiner et al., 2005), it is worth mentioning that it was somewhat surprising to find Cx45 expression in an essentially rodless retina.

On the contrary, the finding that Cx45 is highly expressed by progenitor cells in the chick retina could have been anticipated. Using Cx45 LacZ-reporter mouse, Maxeiner et al. (2003) suggested that Cx45 channels have a rather ubiquitous role during nervous system development. As mentioned earlier, Cx channels participate in essential processes during retinal histogenesis. For example, cell coupling is involved in the establishment of regulatory compartments during programmed cell death (Cusato et al., 2003). Also important is the spontaneous activity that propagates as waves of action potentials in retinal cells, mediated by a combination of chemical and electrical synaptic transmission (Wong, 1999; Firth et al., 2005). To date, the identity of the Cx which mediates such cell–cell interactions in the avian retina remains largely unknown. Taken together, our PCR, immunohistochemistry and Western blot data specified Cx45 as a candidate for a major role in these processes.

Cx50 in the Chick Retina: Completion of Retinal Circuitry?

Although Cx50 gene expression was assessed in mouse (Guldenagel et al., 2000; Kihara et al., 2006), the spatial distribution of this Cx in the retina was mainly studied in the rabbit (Schutte et al., 1998; Huang et al., 2005; O'Brien et al., 2006). In these studies (Huang et al., 2005; O'Brien et al., 2006), Cx50 mRNA and protein were described for the A-horizontal cells. In addition, Cx50 was characterized in macroglia, both in rabbit and rat retina (Schutte et al., 1998). Although few studies describing Cx50 in neural tissue are available, information regarding ontogenetic expression this Cx was very limited, in contrast to studies carried out with the lens (White et al., 1998; Yin et al., 2001; Zhou et al., 2002). In the present study, we observed a specific spatial pattern of Cx50 distribution in the mature chick retina when compared to the description in other species, and also provided a first examination of Cx50 ontogeny in neural tissue.

In contrast to Cx43 and Cx45, we did not detect significant expression of Cx50 at early embryonic ages, but a pronounced upregulation during retinal histogenesis. It is feasible that the increased Cx50 expression, which was mainly observed for late stages of embryonic development (E15), was related to the differentiation and synaptic maturation of retinal cell types that show late development, such as amacrine and bipolar cells. According to this hypothesis, we observed a progressive accumulation of this protein in the inner plexiform layer at the end of embryonic development and in the mature retina. Indeed, we observed

colocalization with CR, indicating the expression of Cx50 by a specific subset of amacrine cells (for review, see Drenhaus et al., 2004). As previously stated, retinal neurogenesis basically takes place in the chick retina within the period comprising E2–E12, but completion of retinal wiring, which involves other processes such as synaptogenesis and the expression synaptic elements, is only achieved later (Prada et al., 1991). In this context, Cx50 expression arises simultaneously to the formation of the basic circuitry underlying visual signaling, depicting a spatial–temporal pattern similar to the pattern observed for neuronal Cx36 in the mouse retina (Degen et al., 2004; Hansen et al., 2005; Kihara et al., 2006).

As a conclusion, Cx ontogenetic expression in the chick retina indicated particular roles for these genes in the mature tissue and simultaneously revealed similarities and differences when compared to other species.

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Figure 1.

Detection of Cx45 using real-time PCR. (A) Amplification plots from cDNA serial dilutions (1:1, 1:3, 1:9, and 1:27) using primers designed for chick Cx45 gene. Notice that no amplification was obtained using Cx56 primers or using Cx45 primers in no template control (NTC). (B) Graph of the first derivative plot from normalized fluorescence against temperature (60–95°C). The melting temperature of PCR products (indicated by the peak of the solid lines) matches exactly to the theoretical T_m expected for the amplicon calculated previously. (C) Linear regression analysis using data from different concentrations of cDNA. $R^2 = 0.99$ indicates a strong correlation between logarithmic estimated cDNA concentration and cycle threshold (CT). Mathematical analysis of linear regression determined the efficiency (E) of the primers ($1 + E = 1.98$), which are close to the expected. Amplicon exponential growth in PCR is theoretically defined as $X_n = X_0 \cdot 2^n$, where X means number of copies and n corresponds to the number of cycles.

Figure 2.

Connexin gene expression during chick retinal histogenesis. (A) Cx43; (B) Cx45; (C) Cx50. Means from embryonic developmental ages (E4.5, E7, E15) were normalized based on the respective adult expression level (P15), and were presented as fold-expression (2^n). (D) GAPDH gene expression was used as internal standard. Whereas Cx45 gene expression did not change during development, Cx43 was highly expressed at E4.5 (67-fold) and E7 retinas (64-fold) when compared to P15. On the other hand, Cx50 was less expressed at E4.5 and E7 retinas (23% and 35%, respectively) when compared to P15. Bars represent standard error of mean. * $p < 0.01$ vs. P15.

Figure 3.

Cx43 immunolabeling (green) in vertical sections of the chick retina counterstained with propidium iodide (red). At E4.5, we found an intense labeling in the outer part of the neuroblastic layer. At E7, Cx43 staining formed a thin line between the neuroblastic layer and the pigmented epithelium. At E12 and E15, we observed the punctate labeling depicting a line in the outer plexiform layer. Also, puncta in the outer part of the outer nuclear layer, in the inner part of the inner nuclear layer and in the ganglion cell layer were seen. At E18 and P15, we observed labeling forming lines in the outer plexiform layer and the inner plexiform

layer, in addition to puncta in the ganglion cell layer. Labels indicate the approximate location of the pigmented epithelium (pe), neuroblastic layer (nbl), outer nuclear layer (onl), outer plexiform layer (opl), inner nuclear layer (inl), inner plexiform layer (ipl), and ganglion cell layer (gcl). Scale bar: 30 μ m.

Figure 4.

Double-labeling experiments in vertical sections of adult chick retina using anti-Cx43 (green) and anti-PAN (red). (A) Cx43 was seen in the inner retina, particularly in the ganglion cell layer (gcl) and in the transition of this layer with the inner plexiform layer (ipl). (B) In the outer margin of ganglion cell layer, anti-PAN, a marker for neuronal processes, labels ganglion cell processes. (C) Superposition of green and red channels using confocal microscopy. In high magnification of selected area (white square, D–F) we clearly observed Cx43 puncta in colocalization with PAN (arrows) and also surrounding presumptive ganglion cell perykarion in the ganglion cell layer (arrowheads). Scale bar: 20 μ m.

Figure 5.

Cx45 immunolabeling (green) in vertical sections of the chick retina counterstained with propidium iodide (red). At E4.5, a ubiquitous distribution of the characteristic Cx punctate immunolabeling was seen in the entire retina. At E7, Cx45 staining was observed especially at the inner part of the neuroblastic layer, and the typical discrete labeling was seen, although these puncta were also present forming aggregates. At E12, Cx45 labeling was seen mainly in the inner plexiform layer, in the ganglion cell layer and in the nerve fiber layer. At E15, a similar distribution was observed, although some immunoreactivity was

occasionally observed in the inner part of the inner nuclear layer. The spatial pattern described for E15 did not significantly change in transverse sections of E18 and postnatal day 15 (P15) retinas. Labels indicate the approximate location of the neuroblastic layer (nbl), outer nuclear layer (onl), outer plexiform layer (opl), inner nuclear layer (inl), inner plexiform layer (ipl), ganglion cell layer (gcl), and nerve fiber layer (nfl). Scale bar: 30 μm .

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Figure 6.

Double-labeling experiments in transverse sections of adult chick retina using anti-Cx45 (green) and anti-GFAP (red). (A) In the chick retina anti-GFAP stains processes of Müller glial cells running vertically throughout the retina. High magnification of selected areas 1 (B, D, F) and 2 (C, E, G) permitted identification of Cx45 and GFAP colocalization (arrows). Within these areas, it is also possible to identify Cx45 punctate which did not colocalize with GFAP (arrowheads). Labels indicate the approximate location of the outer nuclear layer (onl), outer plexiform layer, inner nuclear layer (inl), inner plexiform layer (ipl), ganglion cell layer (gcl), and nerve fiber layer (nfl). Scale bar: 20 μ m.

Figure 7.

Cx50 immunolabeling (green) in transverse sections of the chick retina counterstained with propidium iodide (red). We were not able to detect Cx50 labeling in the neuroblastic layer from 4.5- and 7-day-old embryos (E4.5 and E7, respectively). At E12, Cx50 staining was seen as a faint punctate located mainly in the outer plexiform layer and inner plexiform layer, and also in the transition of the latter with the ganglion cell layer. In E15 retinas, labeling was seen in both outer and inner plexiform layers, and also in the ganglion cell layer and in the inner half of the inner nuclear layer. A similar pattern was observed at E18

and P15, although punctate density seemed to increase at these ages. Labels indicate the approximate location of the neuroblastic layer (nbl), outer nuclear layer (onl), outer plexiform layer (opl), inner nuclear layer (inl), inner plexiform layer (ipl), and ganglion cell layer (gcl). Scale bar: 30 μm .

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Figure 8.

Double-labeling experiments in transverse sections of adult chick retina using anti-Cx50 (green) and anti-calretinin (CR, red). (A) Cx50 punctate labeling was observed within the inner plexiform layer. (B) In the chick retina, anti-CR staining depicts horizontal lines in the inner plexiform layer, corresponding to processes from amacrine cells located in the inner nuclear layer and in the ganglion cell layer. (C) Confocal microscopy revealed that Cx50 colocalizes with CR (arrows), indicating that Cx50 is expressed by amacrine cells. Notice that Cx50 punctate was present in processes and perykaria of CR positive cells. We could also observe Cx50 puncta which did not colocalize with CR immunolabeling (arrowheads). Labels indicate the approximate location of the inner nuclear layer (inl), inner plexiform layer (ipl), and ganglion cell layer (gcl). Scale bar: 30 μ m.

Figure 9.

Semiquantitative analyses of Cx expression during chick histogenesis. (A) Western blots of Cx expression using different antibodies; (B) rabbit anti-Cx43; (C) mouse anti-Cx43; (D) rabbit anti-Cx45; (E) rabbit anti-Cx50. Plots in the graphs express the ratio of optical densitometry (OD) from bands normalized by the respective value found in adult retina (P15) and correspond to the mean from three independent experiments. Arrows indicate the approximate molecular weight of the bands. Bars represent standard error of mean. * $p < 0.05$ vs. P15.

Table 1

Connexin Primers and Antibodies Used in This Study

Gene/Protein (GenBank#)	Primers	Antibody	Antibody Specific
Cx43 (M29003)	Up: 5'-CTGAGTGCCATCTACACCTGTGA-3' Down: 5'-TTGGACGGGACAGGAACA-3'	Zymed 13-8300 Zymed 71-0700	Staining of both antibodies eliminated by peptide preadsorption (5×)
Cx45 (NM 204997)	Up: 5'-CCCGATCAGATGCAATACACA-3' Down: 5'-GGCGATGTGGCTTTGTCT-3'	Gift from Dr. Thomas Steinberg and Dr. Michael Koval	Omission of primary antibody, doublet band located at expected size in WB
Cx50 (aka Cx45.6) (L24799)	Up: 5'-CCGCATTCTCCCTTTAG-3' Down: 5'-TGTTGGCCTGGAGACAAAA-3'	Own production (Dr. Jiang)	IHC and WB positive controls performed in chick lens
Cx56 (L02838)	Up: 5'-AGACACTGTTTGAGGTAGGCTTCA-3' Down: 5'-GCTGCACTGGTAGACTGGCTTT-3'	Own production (Dr. Jiang)	Staining eliminated by peptide preadsorption (5×)
GAPDH (AF047874)	Up: 5'-GGTGCTAAGCGTGTATATCATCTCA-3' Down: 5'-CATGGTTGACACCCATCACAA-3'		
PAN		Zymed 18-0171	Omission of primary antibody, labeling in accordance to previous studies ^a
GFAP		Sigma G3893	Omission of primary antibody, labeling in accordance to previous studies ^b
Calretinin		Chemicon AB1550	Omission of primary antibody, labeling in accordance to previous studies ^c

^aBrocco and Panzetta, 2002; Shin et al., 2003.^bTorelli et al., 1989.^cDrenhaus et al., 2004.