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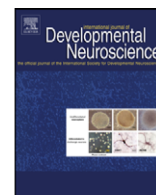

Connexin-mediated communication controls cell proliferation and is essential in retinal histogenesis

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ABSTRACT

Connexin (Cx) channels and hemichannels are involved in essential processes during nervous system development such as apoptosis, propagation of spontaneous activity and interkinetic nuclear movement. In the first part of this study, we extensively characterized Cx gene and protein expression during retinal histogenesis. We observed distinct spatio-temporal patterns among studied Cx and an overriding, ubiquitous presence of Cx45 in progenitor cells. The role of Cx-mediated communication was assessed by using broad-spectrum (carbenoxolone, CBX) and Cx36/Cx50 channel-specific (quinine) blockers. *In vivo* application of CBX, but not quinine, caused remarkable reduction in retinal thickness, suggesting changes in cell proliferation/apoptosis ratio. Indeed, we observed a decreased number of mitotic cells in CBX-injected retinas, with no significant changes in the expression of PCNA, a marker for cells in proliferative state. Taken together, our results pointed a pivotal role of Cx45 in the developing retina. Moreover, this study revealed that Cx-mediated communication is essential in retinal histogenesis, particularly in the control of cell proliferation.

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1. Introduction

In the cell membrane, hexameric arrays of a multigene family of proteins called connexins (Cx) 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 provides intercellular coupling in the gap junctions (GJ), allowing transit of molecules up to 1 kDa, including second messengers, nucleotides and ions (Koval, 2006; Sosinsky and Nicholson, 2005).

In the retina, GJ channels form extensive and plastic networks (Dermietzel et al., 2000; Vaney, 2002; Bloomfield and Volgyi, 2009). Several Cx have been identified in the adult mammalian retina (Condorelli et al., 1998; Guldenagel et al., 2000; Kihara et al., 2003). For example, Cx36 and Cx45 were identified as neuronal Cx participating in the rod-mediated circuitry (Deans et al., 2002; Feigenspan et al., 2001; Maxeiner et al., 2005), whereas Cx57 is apparently restricted to horizontal cells (Hombach et al., 2004). In the adult retina, Cx37 was identified in endothelial cells, and Cx43 is restricted to astrocytes and Müller glial cells (Guldenagel et al., 2000; Zahs et al., 2003).

In addition to the circuitry-related roles attributed in the adult retina (Deans et al., 2002; Feigenspan et al., 2001; Kihara et al., 2006c), Cx-mediated communication participates in processes underlying its development (for review, see Cook and Becker, 2009), such as apoptosis (Cusato et al., 2003), interkinetic nuclear movement (Pearson et al., 2005b) and propagation of spontaneous activity, also known as "retinal waves" (Roerig and Feller, 2000). In this context, GJ channels have been implicated 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; Linden, 2000). Also important is the participation of Cx-mediated communication in the interkinetic nuclear movement. During G1 and G2 phases of the cell cycle, progenitor cell nuclei migrate back-and-forth across the neuroblastic layer, and cells undergo mitosis only in a region immediately adjacent to the retinal pigmented epithelium, termed ventricular zone. Indeed, recent data indicated that disruption of

Abbreviations: BSA, bovine serum albumin; CB, calbindin-D; CBX, carbenoxolone; Cx, connexin; GFAP, glial fibrillary acidic protein; GJ, gap junction; kDa, kilo Dalton; Ki67, antigen identified by monoclonal antibody Ki-67; PB, phosphate buffer; PCNA, proliferating cell nuclear antigen.

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Cx-mediated communication acts to slow this nuclear movement (Pearson et al., 2005b). Finally, another essential process in retinal histogenesis is the spontaneous activity that propagates as waves of action potentials, driving rhythmic increases of intracellular calcium in retinal neurons, mediated by a combination of chemical and electrical synaptic transmission (Firth et al., 2005; Wong, 1999).

It is well established that all these events are essential in retinal histogenesis, although the identity of Cx(s) participating in these processes remains unclear. In this context, the first aim of this study was to extensively characterize Cx expression during the development of the retina. Secondly, we disrupted Cx-mediated communication in order to retrieve its functional role in retinal histogenesis. In this regard, RNAi and KO strategies consist in useful tools, but they can also be problematic, since compensatory, downstream changes sometimes occur during development (Cruikshank et al., 2004; De Zeeuw et al., 2003). Thus, we relied on a more direct approach, using pharmacological blockers that (1) have broad-spectrum efficacy on GJ channels formed by different Cx, or (2) act on specific Cx, without major effects on other GJs or membrane channels. The effect of these blockers in the propagation of apoptotic activity (Cusato et al., 2003) and retinal waves (Singer et al., 2001; Hansen et al., 2005) was determined in previous studies. Third, we analyzed how disruption of Cx-mediated communication specifically affects essential processes in histogenesis, such as cell cycle control and proliferation.

2. Materials and methods

2.1. Animal procedures

Experiments were carried out with Wistar rats (*rattus novergicus*) kept on a 12 h light/dark cycle (light phase 80–100 lx) with lights on at 06:00 a.m. Embryonic day 19 (E19) and postnatal day 1 (P1), P5, P10, P15 and P60 rats were sacrificed with an overdose of ketamine (30 mg/100 g of body weight, i.m., Parke-Davis, Ann Arbor, MI, USA) and xylazine (2 mg/100 g, i.m., West Haven, CT, USA) between 10:00 a.m. and 12:00 a.m. Next, the retinas were removed for different methodologies. All experiments were conducted in accordance to the NIH and the Biomedical Sciences Institute/USP guidelines.

2.2. RNA isolation, cDNA synthesis and real-time PCR

Retinas were directly homogenized in 1 ml TRIzol reagent (Invitrogen) and total RNA was extracted following manufacturer's suggested protocol (Kihara et al., 2005). In brief, 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 obtained from A_{260} measurements. Residual DNA was removed using DNase I (Amersham, Piscataway, NJ, USA) following manufacturer's protocol. For each 20 μl reverse transcription reaction, 2 μg total RNA was mixed with 1 μl oligodT primer (0.5 μg; Invitrogen) and incubated for 10 min at 65 °C. After cooling on ice the solution was mixed with 4 μl 5× first strand buffer, 2 μl of 0.1 M DTT, 1 μl of dATP, dTTP, dCTP and dGTP (each 10 mM), and 1 μl 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. Rotor-Gene 6000 Real-Time Rotary Analyzer (Corbett Robotics Inc., San Francisco, USA) with specific primers for different connexins (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 °C to 95 °C, in order to evaluate primer specificity.

2.3. PCR data and statistical analysis

Quantification of gene amplification was performed by determining the cycle threshold (CT) based on the fluorescence detected within the geometric region of the semi-log view of the amplification plot. An amplification plot for each sample was generated showing the increase in reporter dye fluorescence (ΔRn) with each cycle of PCR. From each amplification plot a CT value was calculated, representing the PCR cycle number at which the fluorescence was detectable above an arbitrary threshold, based on the variability of baseline data in the first fifteen cycles. Relative quantification of target gene expression was evaluated using the comparative CT method as previously described in detail (Belmonte et al., 2006; Medhurst et al., 2000). The ΔCT value was determined by subtracting the target CT of each sample from its respective GAPDH CT value, used as internal control. Calculation of ΔΔCT involves using the Control group mean ΔCT value as an arbitrary constant to subtract from all other ΔCT mean values. Fold-changes in gene expression of the target gene are equivalent to $2^{-\Delta\Delta CT}$. 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%.

2.4. Immunohistochemistry

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 O.C.T. compound they were cut transversally (12 μm) 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.

As described in Table 1, we used a polyclonal antibody raised against amino acids 296–304 of human Cx36 (36-4600, Zymed/Invitrogen), whose specificity was determined in retinas of Cx36 KO (Kihara et al., 2006a). To identify Cx43, we used a polyclonal antibody against amino acids 251–270 of rat Cx43 (71-0700, Zymed/Invitrogen), which has been employed in rodent retinal studies (Kihara et al., 2006b; Zahs et al., 2003). Specificity of this antibody was previously determined in $Cx43^{+/-}$ deficient mouse in addition to peptide pre-adsorption controls (Kihara et al., 2006b). To investigate Cx45 protein distribution, two different antibodies were utilized: a monoclonal antibody (MAB3101, Chemicon, Temecula, CA) and a polyclonal antibody (AB1745, Chemicon), both raised against a peptide corresponding to amino acids 354–367 of human Cx45 and that does not possess sequence homology with Cx43 (Coppen et al., 1998). These Cx45 antibodies have been used in mouse retinal studies (Petrasch-Parwez et al., 2004). Staining of both antibodies was eliminated in pre-adsorption controls performed in mouse retina (Kihara et al., 2006b). Cx57 expression was investigated using a rabbit polyclonal antibody raised against a synthetic peptide derived from an internal region of the mouse Cx57 protein (40-5000, Zymed/Invitrogen). In Western blots, this antibody identifies the target bands at 57 and 64 kDa; the 64 kDa band likely represents a post-transcriptional modified form of Cx57, consistent with the multiple phosphorylation sites of this protein (manufacturer information). To detect and characterize cell proliferation, we used anti-Ki67 (VP-RM04, Vector Labs, Burlingame, CA, USA). Ki67 is a cell cycle associated protein expressed from G1 through the end of M phase, which has been extensively used as a cell proliferation marker. In the retina, its expression has been associated with mitotic cells (Ikeda et al., 2005).

Some double-labeling experiments were also carried out using one of the antibodies against connexins and another against glial fibrillary acidic protein (GFAP) or calbindin (CB), as markers for astrocytes (Guldenagel et al., 2000) and horizontal cells (Haverkamp and Wassle, 2000; Kihara et al., 2008b), respectively. A monoclonal antibody raised against GFAP from pig spinal cord (mouse anti-GFAP, Sigma, catalog # G3893, lot # 082K4834) was used (1:2000–1:4000). This anti-GFAP stains a single band of 50 kDa molecular weight on Western blot (manufacturer's technical information) and provided a pattern of cellular morphology and distribution as previously described in mouse retinal studies (Sohl et al., 2000). A monoclonal antibody raised against chick calbindin-D (mouse anti-calbindin-D, Sigma, catalog # C8666, lot # 28H4845) was used (1:250–1:500), which stains a band of 28 kDa on two-dimensional immunoblot (manufacturer's technical information).

After several washes, retinal sections were incubated with goat antiserum against rabbit or mouse IgG tagged to Alexa™ 488 (Molecular Probes, Eugene, Oregon, 1:500–1:1000) or fluorescein isothiocyanate (FITC, Jackson Labs, West Grove, PA, 1:100–1:500) diluted in PB containing 0.5% Triton X-100 for 2 h at room temperature. For double-labeling experiments, secondary antibodies conjugated tetramethyl rhodamine isothiocyanate (TRITC, Jackson Labs, 1:500–1:1000) and to indodicarbocyanine (Cy5, Jackson Labs, 1:500, 1:1000) were used. In order to better visualize Cx labeling, we counter-stained retinas using propidium iodide, by incubating sections at room temperature for 30 min. After washing, the tissue was mounted using Vecta Shield (Vector Labs, Burlingame, CA).

2.5. Image acquisition and quantification

Single scan images (1024 × 1024 pixels) were acquired by using a Nikon PCM2000 (Nikon Corporation, Tokyo, Japan) microscope confocal scanning system. Quantification of double-labeling experiments was described previously (Kihara et al., 2009). After channel separation (RGB) of color images, we performed computational colocalization analysis. Our confocal scanning system generated color images, and the software determined the colocalization coefficient from different RGB channels, which indicates the relative degree of overlap between signals. Figures were mounted with Adobe Photoshop CS. Manipulation of the images was restricted to threshold and brightness adjustments to the whole image.

2.6. Western blotting

As previously described (Santos-Bredariol et al., 2006), retinas were rapidly dissected, washed with PBS, and homogenized in 25 mM Tris buffer containing a cocktail of protease inhibitors (Calbiochem, La Jolla, CA, USA). Homogenates were

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