Expression of pannexin family of proteins in the retina

Galina Dvoriantchikova\textsuperscript{a}, Dmitry Ivanov\textsuperscript{a,b}, Yuri Panchin\textsuperscript{c,d}, Valery I. Shestopalov\textsuperscript{a,e,*}

\textsuperscript{a} Bascom Palmer Eye Institute, Department of Ophthalmology, University of Miami Miller School of Medicine, 1638 NW 10th Avenue, Miami, FL 33136, USA
\textsuperscript{b} Vavilov Institute of General Genetics, RAS, Moscow, Russia
\textsuperscript{c} Institute for Information Transmission Problems, RAS, Moscow, Russia
\textsuperscript{d} A.N. Belozersky Institute, Moscow State University, Moscow, Russia
\textsuperscript{e} Department of Cell Biology and Anatomy, University of Miami Miller, School of Medicine, Miami, FL, USA

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\begin{abstract}
Expression of the Panx1 and Panx2 members of the pannexin family of gap junction proteins was studied in the retina by in situ hybridization and qRT-PCR. Both pannexins showed robust expression across the retina with predominant accumulation in the retinal ganglion cells (RGCs). In concordance, immunohistochemical analysis showed accumulation of the Panx1 protein in RGCs, amacrine, horizontal cells and their processes. Two Panx1 isoforms were detected: a ubiquitously expressed 58 kDa protein, and a 43 kDa isoform that specifically accumulated in the retina and brain. Our results indicated that Panx1 and Panx2 are abundantly expressed in the retina, and may therefore contribute to the electrical and metabolic coupling, or to signaling between retinal neurons via the secondary messengers.
\end{abstract}

\begin{keywords}
Pannexin family; Gap junction proteins; Retina; Retinal ganglion cells; Gene expression; Antibody
\end{keywords}

1. Introduction

Neurons in the vertebrate retina are electrically and metabolically coupled into multiple networks via cell type-specific gap junctions formed by selective expression of several connexins [1]. Currently, connexins are suggested as the sole conduits for gap junctions formed by selective expression of several connexins, although other authors retain the prefer to use term pannexins both for vertebrate and invertebrate gap junction proteins, although other authors retain term “innexin” for invertebrate species. Recently, the rodent Panx1, alone and in combination with Panx2, was demonstrated to induce formation of the intercellular channels in paired oocytes [6]. Although Panx2 does not form homotypic channels [6], it might participate in heterotypic channel formation, providing a modulatory effect on Panx1 channels. Significantly, Panx2 expression is restricted to central nervous system (CNS), thus suggesting that the modulation of heterotypic channel properties is required for neuron-specific functions [4,6,7]. When expressed in single Xenopus oocytes, Panx1 hemichannels were shown to be functional in plasma membranes [8]. This type of non-junctional function has been reported earlier for connexins [9]. Presently, it is not clear, whether pannexins simply duplicate connexins’ gap junction functions or play a distinct physiological role. More recent analysis of Panx1 hemichannel properties demonstrated their mechanosensitivity, the ability to open at physiological concentrations of Ca\textsuperscript{2+} and pass signaling molecules like ATP [8]. Hemichannels have been shown to control a negative feedback pathway between horizontal cells and cone photoreceptors [10]. These findings suggested a potential contribution of pannexins to electrical and metabolic coupling, and a role in secondary messenger communication between cells, properties, which might be indispensable in highly interconnected tissues like retina. In this work, we characterized the expression and localization pattern of Panx1 and Panx2 in the retina that might provide insights into their function in CNS.

2. Materials and methods

2.1. Animals, cell lines and plasmids

All experiments were performed in compliance with the ARVO statement for use of animals in ophthalmic and vision research. C57BL/6 mice and brown Norway rats (\textit{Rattus norvegicus}) were housed in animal care facilities according to NIH guidelines and University of Miami IACUC approved protocols. P5, P20 and adult mouse and adult rat tissues were used for qRT-PCR analysis, in situ hybridization, RGCs isolation by immunopanning, Western blot and immunohistochemistry (IHC) analysis. Animals were euthanized by CO\textsubscript{2} inhalation according to the IACUC approved protocol. Retina, brain, heart and skeletal muscle tissues for analysis were collected under University of Miami IACUC approved protocols. P5, P20 and adult mouse and adult rat tissues were used for qRT-PCR analysis, in situ hybridization, RGCs isolation by immunopanning, Western blot and immunohistochemistry (IHC) analysis. Animals were euthanized by CO\textsubscript{2} inhalation according to the IACUC approved protocol. Retina, brain, heart and skeletal muscle tissues for analysis were collected immediately after euthanasia. Human bone marrow endothelial (HBME) cells were maintained in DMEM with 10% FBS at 37 °C with 5% CO\textsubscript{2}. HBME cells were transfected using a GeneJammer Transfection Reagent (Stratagene, La Jolla, CA). We used pEGFP-N1 plasmid vector (Invitrogen, Carlsbad, CA) and pEGFP-Panx1 (gift S. Lukyanov) construct based on the same vector backbone and expressing c-terminal fusion protein with Panx1 for cell line transfections.

2.2. Purification of retinal ganglion cells and RNA extraction

To detect levels of the RGC-specific accumulation of transcripts we purified RGCs using immunopanning method reported earlier [11]. In brief, retinas from enucleated rats eyes were mechanically dissected...
under a microscope followed by incubation in a papain solution (16.5 U/ml) for 30 min until full dissociation. After one round of negative selection against macrophages and endothelial cells (using anti-macrophage antiserum, Axell Accurate Chemical Corp, Westbury, NY), RGCs were panned using anti-Thy1.1 antibody (cell line T11D7e2, ATCC). RGCs were released by 0.125% trypsin solution in EBSS (Invitrogen), collected and lysed in the Absolutely RNA**/C210 Nanoprep kit (Stratagene, La Jolla, CA), utilized for RNA extraction.

2.3. Antibodies

Chicken polyclonal anti-GFP antibodies were purchased from (Aves Labs, OR). To prepare a rabbit polyclonal antibody against the carboxyl terminus of human PANX1, cDNA encompassing the entire coding region was synthesized by PCR amplification of the cDNA insert from clone IMAGE: 4390851 using two gene-specific primers (Px1F: 5'-TCTGGATCCTACACGCTGTTTGTTCCA-3'; Px1R: 5'-TCTAAGCTTGCAAGAAGAATCCAGAAG-3') and was inserted into the BamHI and HindIII sites of pET-23a to yield the pETPx1 plasmid. The resultant 18 kDa pETPx1 protein fused to the His-tagged C-terminus was purified from E. coli and used for immunization of the rabbit. The affinity purified rabbit serum containing specific anti-human PANX1 activity and lacking any significant non-specific activities was used for Western blot and IHC applications.

For experiments using qRT-PCR, in situ hybridization, Western blot, amino acid sequence analysis, retrograde labeling of RGCs and immunocytochemistry we applied standard techniques, brief protocols are included in the Supplement 1.

3. Results and discussion

3.1. Panx1 and Panx2 expression in the retina

To determine the expression of genes encoding Panx1 and Panx2 across the retina we used in situ hybridization. Transcripts for Panx1 and Panx2 were detected in the ganglion cell layer (GCL) and across the retina (Fig. 1). No staining was observed with the sense control probes supporting the specificity of staining for the Panx1- and Panx2-probes (data not shown). RGCs are located in the GCL of the retina and are easily distinguishable from satellite glial cells due to their relatively large round cell bodies with asymmetrically positioned nuclei [12]. DIG-labeled cell bodies had localization and morphology characteristic to that of RGCs, and were evenly spaced across the retina, indicating that RGCs were positive for Panx1 and Panx2 expression (Fig. 1). These results are in good agreement with the published data on Panx1 expression in the retina [13].

The levels of accumulation of the transcripts in RGCs were detected by qRT-PCR. In good agreement with the in situ hybridization data, the qRT-PCR data showed an increase in accumulation of Panx1 and Panx2 mRNA in the purified RGCs relatively to that in the whole retina (Fig. 2). The data normalized to the levels of β-actin mRNA revealed consistent enrichment in the Panx2 transcript relative to Panx1 in the adult rat RGCs (Fig. 2).

3.2. PANX1 characterization with antibodies

To investigate the role of PANX1 in the retina we generated rabbit polyclonal anti-PANX1 antibodies using the conserved 20 kDa C-terminal region of the human PANX1 as an antigen (see Section 2). As a result, our antibodies showed cross-specificity with rat and mouse Panx1, which was required for experiments with the rodent PANX1 orthologues. Control tests for antibody specificity and absence of non-specific binding were performed with the HBME cells, that tested negative for Panx1 mRNA by RT-PCR in our preliminary studies. We

Fig. 1. In situ hybridization of paraffin sections of neonatal (P5) retinas using DIG-labeled probes. Antisense strand (A,C) hybridization revealed Panx1 and Panx2 transcripts in the ganglion cell layer (GCL), inner nuclear layer (INL) and scattered across the rest of the retina. PRL, photoreceptor layer. Control hybridization with sense strand (B,D) showed no specific signal. The bar is 100 μm.

Fig. 2. Quantitative RT-PCR analysis of Panx1 and Panx2 gene expression in the retina and purified RGCs. (A) The micrograph of the gel-separated PCR products correspondent to Panx1, Panx2 and control genes, Thy1, Actb. (B) Relative enrichment of Panx1 and Panx2 transcripts after normalization to the levels of Actb.
transfected these cells with the pEGFP–Panx1 construct encoding a fusion protein and probed them on Western blots with anti-Panx1 and anti-GFP antibodies. A specific band of about 75 kDa correspondent to the predicted MW of the fusion EGFP–PANX1 protein was detected only in extracts from the transfected HBME cells by both antibodies (Fig. 3). No specific labeling was detected in untransfected HBME cells and in control blots probed with rabbit pre-immune serum instead of antibodies (data not shown). A minor band of approximately 85 kDa, recognized by both antibodies, was likely a post-translationally modified isoform of EGFP–Panx1. Extract from control cells transfected with pEGFP-N1 vector was recognized by anti-GFP antibodies only, proving the absence of specificity to GFP in our antibodies (Fig. 3). Immunostaining of HBME cells with anti-PANX1 antibodies selectively labeled only the transfected, EGFP-positive cells, but not the untransfected, EGFP-negative cells that were present in the same preparations (Fig. 4). Given our antibodies had no cross-reactivity with EGFP, this result confirmed high specificity of antibodies and lack of non-specific binding. Co-localization of anti-PANX1 immunostaining with the EGFP fluorescence was obvious in organelles, most likely in the endoplasmic reticulum (ER) and Golgi. Such labeling pattern is typical for tetraspanins, like nascent connexins, en route to the plasma membrane and could be more pronounced with fusion proteins, which are often processed less efficiently then the endogenous protein [14].

To study the Panx1 isoforms expressed in the retina, we prepared organelle-enriched (OR) and plasma membrane-enriched (PM) fractions (see Materials and Methods, Supplement) using protein extracts from retina brain, heart and skeletal muscles, tissues shown to express Panx1 at high levels [4]. Western blot analysis revealed two distinct isoforms of Panx1: 43 kDa protein was present in both OR fractions of brain and retina (lanes 1–5, Fig. 5), but was not detected in skeletal and heart muscles (lanes 6–9); 58 kDa isoform was ubiquitously present in PM (lanes 2, 4, 6, and 8) and depleted in OR fractions of all tissues examined (lanes 3, 5, and 7). The third, minor isoform had a MW of 90 kDa corresponding to the size of the Panx1 homodimer and was detected in all OR fractions. The fact that 58 kDa isoform was quantitatively sedimented at the low (7500 × g) speed centrifugation, strongly suggest this isoform is associated with insoluble cellular fractions, most likely with plasma membrane. In contrast, the 43 kDa isoform was present in OR fractions and is likely associated with organelles, which form microsomes that could not be pelleted at 7500 × g [15]. While the 43 kDa isoform closely matches the predicted size of Panx1 reported earlier [16]; the larger 58 kDa is likely post-transla-
tional modification of the protein. Phosphorylation that significantly increases MW of the protein has been shown with connexins [17]; glycosylation is another typical type of post-translational modifications of tetraspanin proteins [18]. Amino acid sequence analysis data failed to predict O-glycosylation sites, but predicted one N-glycosylation site in position 337aa and multiple phosphorylation sites (12 sites for serine, one site for threonine and four sites for tyrosine phosphorylation), supporting this possibility (see Supplement 1.6).

3.3. Panx1 protein localization in the retina

Cellular distribution of Panx1 was examined by IHC in fixed rodent retinas. As shown in Fig. 6, prominent Panx1-specific immunostaining localized to somata and processes of ganglion (Fig. 6A–C), amacrine and horizontal cells (Fig. 6B and C) in mouse retina. Similar pattern was observed in juvenile animals, but the overall Panx1 accumulation reduced significantly in adult retinas, persisting mostly in RGCs. These results confirmed the qRT-PCR data on the Panx1 transcript enrichment in RGCs relative to other retinal cells. To verify RGC-specificity of Panx1 labeling, we analyzed Panx1 immunostaining (Fig. 7A) in rat retinas that had RGCs retrogradely labeled by the lipophillic dye 4DI-10ASP (Fig. 7B). In these retinas, 4DI-10ASP, which specifically labels RGC [19], in cytoplasmic organelles, plasma membranes and processes. These experiments revealed that 100% of the 4DI-10ASP-labeled cells were Panx1-positive (Fig. 7). Significantly, Panx1-specific punctate labeling was observed in the processes of RGCs intermingled and partially co-localized with the 4DI-10ASP labeling of the RGC processes in the IPL (arrows on Fig. 7). This region is known to contain neuronal connexin-mediated electrical contacts [20], therefore it was logical that Panx1-mediated junctional contacts also localized there.

Horizontal cells that express Panx1 and can be easily distinguished by morphology and localization in the OPL, are second-order neurons, which are post-synaptic to photoreceptors and are well known for the electrical and dye coupling interconnecting them into the network [1,21]. However, in the Cx57 knockout mouse the dye coupling between horizontal cells become completely disrupted, hinting that Panx1 function in these neurons might be different from providing a gap junction-like coupling [21]. Based on the published properties of the Panx1 hemichannels [8,22], we speculate that they may rep...
resent a conduit for a negative feedback pathway between horizontal cells and cone photoreceptors, an essential feature of horizontal cells, the molecular nature of which remains unclear [10]. At a sub-cellular level, Panx1-specific labeling was evident in the processes as well as in somata, localizing to organelles in the perinuclear region of the RGC cytoplasm and in horizontal cells. Relatively little plasma membrane labeling was observed in somata of neonatal RGCs, gradually increasing with age (insets, Fig. 6). The perinuclear labeling is consistent with the pattern observed in the EGFP-PANX1-transfected HBME cells. Other evidence of organelle localization of Panx1, provided by co-localization of Panx1 and lipophilic 4Di-10ASP dye and by the Western blot data, were discussed above. Localization to organelles might indicate that, similar to connexins [14,23], Panx1 utilizes an ER–Golgi trafficking route for the newly synthesized protein. Alternatively, the 43 kDa organelle-bound isoform might have an organelle-specific function in the membranes of the ER and Golgi. Recent studies suggesting that Panx1 hemichannels play a role in regulating Ca+ homeostasis and release from the ER stores (R. Skryma, personal communication), support this possibility. Our results indicate that plasma membrane-bound Panx1 labels mostly neuronal processes in retinal neurons, the rest of the protein labels organelles.

In this work, we characterized the expression and the protein intercellular localization of Panx1 and Panx2 in different types of neuron in neonatal and adult rodent retinas that might provide insights into their function in the CNS. Our results suggest that pannexins might play a role in electrical and metabolic coupling and a role in secondary messenger communication in neuronal networks.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2006.03.026.

References