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COMPLEXITY OF GAP JUNCTIONS BETWEEN HORIZONTAL CELLS OF THE CARP RETINA

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Abstract—In the vertebrate retina, horizontal cells (HCs) reveal homologous coupling by gap junctions (gj), which are thought to consist of different connexins (Cx). However, recent studies in mouse, rabbit and zebrafish retina indicate that individual HCs express more than one connexin. To provide further insights into the composition of gj connecting HCs and to determine whether HCs express multiple connexins, we examined the molecular identity and distribution of gj between HCs of the carp retina. We have cloned four carp connexins designated Cx49.5, Cx55.5, Cx52.6 and Cx53.8 with a close relationship to connexins previously reported in HCs of mouse, rabbit and zebrafish, respectively. Using *in situ* hybridization, Cx49.5 expression was detected in different subpopulations of retinal neurons including HCs, whereas the Cx52.6 transcript was localized exclusively in HCs. Using specific antibodies, Cx55.5 and Cx53.8 were detected on dendrites of all four HC subtypes and axon terminals. Immunoelectron microscopy confirmed the presence of Cx55.5 and Cx53.8 in gap junctions between these processes and Cx55.5 was additionally observed in HC dendrites invaginating cone pedicles, suggesting its participation in the modulation of photoreceptor output in the carp retina. Furthermore, using single-cell RT–PCR, all four connexins were detected in different subtypes of HCs, suggesting overlapping expression patterns. Thus, the composition of gj mediating homologous coupling between subtypes of carp HCs appears to be more complex than expected. Moreover, BLAST searches of the preliminary carp genome, using novel sequences as query, suggest that most of the analyzed connexin genes are duplicated in carp.

Key words: Connexin49.5, Connexin52.6, Connexin55.5, Connexin53.8, electrical synapse, hemichannel.

INTRODUCTION

Gap junctions are clusters of intercellular channels composed of two hexameric assemblies (hemichannels) of connexins (Cx) that allow direct electrical and metabolic communication between adjacent cells (reviewed in Goodenough et al., 1996; Kumar and Gilula, 1996). Gap junction-mediated intercellular communication exists in nearly all tissues in all vertebrate species. In the retina, the vast majority of cell types are coupled by gap junctions, emphasizing their crucial role in transmission and processing of visual information (reviewed in Völgyi et al., 2013). For example, horizontal cells (HCs), inhibitory interneurons in the outer retina, are extensively coupled, resulting in large receptive fields that allow capturing and integrating inputs from a larger set of photoreceptors. The subsequent modulation of photoreceptors outputs via feedback inhibition contributes to the receptive field organization of bipolar and ganglion cells and mediates color-opponency of retinal cells, thereby optimizing the visual acuity of the retina (reviewed in Thoreson and Mangel, 2012).

The vertebrate retina possesses up to four morphologically distinct types of HCs (Stell and Lightfoot, 1975; Weiler, 1978; Dacheux and Raviola, 1982; Peichl and González-Soriano, 1994). In the zebrafish even six physiologically different types of HCs have been described (Li et al., 2009; Connaughton and Nelson, 2010). HCs of the same functional type are interconnected via gap junctions (reviewed in Vaney, 1994; Bloomfield et al., 1995), resulting in the formation of independent networks, which are very dynamic and differentially modifiable by neuromodulators and second messengers (Umino et al., 1991; Pottek et al., 1997; Weiler et al., 1999, 2000; Xin and Bloomfield, 1999, 2000; He et al., 2000). To understand how exactly gap junction channels contribute to the formation and

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Abbreviations: aa, amino acid; bp, base pairs; Cx, connexin; cc, *Cyprinus carpio*; Da, Dalton; dr, *Danio rerio*; GCL, ganglion cell layer; hs, *Homo sapiens*; HC, horizontal cell; INL, inner nuclear layer; IPL, inner plexiform layer; mm, *Mus musculus*; N2a, neuroblastoma 2a; oc, *Oryctolagus cuniculus*; ONL, outer nuclear layer; OPL, outer plexiform layer.

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functioning of these networks, the unambiguous identification of their constituents is required.

In this context, several connexins have been identified in HCs of different species. However, there are controversies concerning the number and the distribution of connexins expressed in these cells. In the rabbit retina, which contains two types of HCs, gap junctions connecting axonless A-type HCs are composed of Cx50 (O'Brien et al., 2006), whereas the axon terminals of the axon-bearing B-type HCs are coupled via gap junctions composed of Cx57 (Pan et al., 2012). Interestingly, neither Cx50 nor Cx57 is present at the dendro-dendritic gap junctions of axon-bearing HCs in rabbit, leading Pan et al. (2012) to suppose the expression of an additional yet unknown connexin in this HC type. Further support for the expression of multiple connexins in different types of HCs and even different compartments of the same HC comes from studies of the mouse retina. Here, only one type of HC (B-type) exists, in which Cx57 was found in gap junctions between both, dendrites and axon terminal processes (Hombach et al., 2004; Janssen-Bienhold et al., 2009). In addition, Cx50 was localized only in gap junctions involved in the formation of the axon terminal plexus, thus providing evidence for the existence of molecular distinct and independent sets of gap junctions within the same HC compartment (Dorgau et al., 2015). In the zebrafish retina, which possesses four morphologically distinct HC types, recent studies suggested that gap junctions between H1/2 HCs consist of both Cx55.5 and Cx52.6 (Shields et al., 2007; Klaassen et al., 2011; Sun et al., 2012). Furthermore, an additional connexin, Cx52.9, was found to colocalize partly with both Cx55.5 and Cx52.6 in the same gap-junctional plaques (Klaassen et al., 2011), but there is no definite assignment of their expression to a particular HC type. However, these results also point to the existence of multiple connexins involved in the formation of HCs networks in zebrafish.

Here, we aimed to provide further insights into the composition of gap junctions connecting HCs. In this respect, the focus of the present study was to identify potential candidates involved in gap junction formation between different HC types within the carp retina and to determine whether their expression can be assigned to particular HC types.

EXPERIMENTAL PROCEDURES

Animals and tissue preparation

Adult mirror carp (*Cyprinus carpio*) of 10–20 cm body length was used in this study ($n = 23$). Animals were obtained from a local breeding station and kept in an aerated tank under a 12-h dark/light cycle. All experimental procedures were carried out in accordance with the guidelines of the local animal care committee (*Niedersächsisches Landesamt fuer Verbraucherschutz und Lebensmittelsicherheit/LAVES*) and the guidelines of the German Animal Protection Law (*Tierschutzgesetz; BGBl. I S. 1206, 1313 and BGBl. I S. 1934*). Animals were killed rapidly by decapitation and posterior eyecups were dissected either in PBS (pH 7.4) or in Leibovitz's L-15 medium (Sigma, Munich, Germany).

For immunohistochemistry (IHC) and *in situ* hybridization (ISH) eyecups ($n = 6$ for IHC and ISH, respectively) were immersion-fixed in 2% paraformaldehyde (PFA) in 0.1 M phosphate buffer (PB, pH 7.4) for 20 min, cryoprotected with 30% sucrose in 0.1 M PB overnight at 4 °C and embedded in Tissue Tek O.C.T. compound (Sakura Finete Europe, Alphen aan den Rijn, Netherlands). Vertical sections (20 μm) were prepared using a cryostat (LeicaCM1860, Leica Biosystems, Nussloch, Germany) and mounted on gelatine-coated microscope slides (for IHC) or Superfrost Plus microscope slides (for ISH; Menzel, Braunschweig, Germany).

For immunoelectron microscopy, dissociation of retinal neurons, extraction of total retinal RNA and western blot analysis, carp were dark-adapted for at least 1 h and retinas were isolated from the eyecups. For immunoelectron microscopy, isolated retinas ($n = 6$ for the localization of Cx55.5 and Cx53.8, respectively) were fixed in 2% PFA/ 3% sucrose in PB for 30 min, cryoprotected in 30% sucrose in PB overnight and embedded in Tissue Tek. Tangential and vertical sections (60 μm) were prepared using a cryostat.

For dissociation of retinal neurons, isolated retinas ($n = 6$ for single-cell RT–PCR and IHC of isolated HCs, respectively) were incubated in L-15 medium containing 1 mg/ml cysteine-activated papain (20 units/mg, Sigma) for 15 min at room temperature. The enzyme activity was stopped by incubating retinas in L-15 supplemented with 5% fetal calf serum (Biochrom, Berlin, Germany) for 10 min. After several rinses with unmodified L-15 medium, retinas were mechanically dissociated by gentle trituration with fire-polished pasteur pipettes and the cell suspension was placed on glass slides (for IHC of isolated HCs) or cover slips (for RNA extraction from single cells) coated with 50 μg/ml poly-L-lysine (Sigma) and allowed to attach for 1 h.

Cloning of connexin cDNA from carp retina using RT–PCR

Total RNA was extracted from isolated carp retina ($n = 6$) using NucleoSpin® RNA II kit (Macherey Nagel, Düren, Germany) according to the manufacturer's manual. Additional DNaseI (Amplification Grade; Invitrogen, Darmstadt, Germany) treatment was performed according to the manufacturer's instructions to eliminate residual genomic DNA. The first-strand cDNA synthesis was carried out in a final volume of 25 μl containing DNaseI-treated total RNA (1 μg) or RNA from single HCs, oligo(dT)15 primers (0.5 μg; Promega, Mannheim, Germany), random primers (0.05 μg; Promega), dNTPs (0.4 mM each, Carl Roth, Karlsruhe, Germany), RNasin (20 U; Promega), first-strand buffer (1x; Invitrogen), 1,4-dithiothreitol (DTT, 0.5 mM; Invitrogen) and SuperScript® III reverse transcriptase (200 U; Invitrogen), according to the manufacturer's instructions. The integrity and the quality of the cDNA were confirmed via β-actin PCR employing an intron-spanning primer set (bact1 and bact2). All primers used in the