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LACK OF PHOTORECEPTOR SIGNALING ALTERS THE EXPRESSION OF SPECIFIC SYNAPTIC PROTEINS IN THE RETINA

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Abstract—Synaptic modulation by activity-dependent changes constitutes a cellular mechanism for neuronal plasticity. However, it is not clear how the complete lack of neuronal signaling specifically affects elements involved in the communication between neurons. In the retina, it is now well established that both chemical and electrical synapses are essential to mediate the transmission of visual signaling triggered by the photoreceptors. In this study, we compared the expression of synaptic proteins in the retinas of wild-type (WT) vs. rd/rd mice, an animal model that displays inherited and specific ablation of photoreceptors caused by a mutation in the gene encoding the β-subunit of rod cGMP-phosphodiesterase (*Pde6b*^{rd1}). We specifically examined the expression of connexins (Cx), the proteins that form the gap junction channels of electrical synapses, in addition to synaptophysin and synapsin I, which are involved in the release of neurotransmitters at chemical synapses. Our results revealed that Cx36 gene expression levels are lower in the retinas of rd/rd when compared with WT. Confocal analysis indicated that Cx36 immunolabeling almost disappeared in the outer plexiform layer without significant changes in protein distribution within the inner plexiform layer of rd/rd retinas. Likewise, synaptophysin expression remarkably decreased in the outer plexiform layer of rd/rd retinas, and this down-regulation was also associated with diminished transcript levels. Furthermore, we observed down-regulation of Cx57 gene expression in rd/rd retinas when compared with WT and also changes in protein distribution. Interestingly, Cx45 and synapsin I expression in rd/rd retinas showed no noticeable changes when compared with WT. Taken together, our results revealed that the loss of photoreceptors leads to decreased expression of some synaptic proteins. More importantly, this study provides evidence that neuronal activity regulates, but is not essential to maintain, the expression of synaptic elements.

Key words: plasticity, synapse, connexin, synaptophysin, synapsin, retina.

Synaptic plasticity is usually defined as the ability of the connection between neurons to modulate its strength (Sheng and Kim, 2002; Blitz et al., 2004). There are several mechanisms participating in the modulation of a given synapse, including its own activity (Faber et al., 1991; Abarbanel et al., 2002). Classically, electrical coupling provided by gap junctions (GJ) was considered "not plastic," although being important for tuning network activity (Traub et al., 2001). GJ channels couple adjacent cells, allowing transfer of second messengers, ions and molecules up to 1 kDa (for review, see Rozental et al., 2000). These channels are composed by a multigene family of integral membrane proteins called connexins (Cxs), with 20 members in the murine genome (Willecke et al., 2002; Sohl et al., 2004). Hexameric association of Cxs forms a hemichannel (connexon), and the docking of two connexons in apposed cell membranes forms a functional GJ channel. It has been proposed that in addition to form channels in the GJ, hemichannels also function in nonjunctional membranes in the absence of pairing with partners from adjacent cells, although this matter remains under debate (Jiang and Gu, 2005; Spray et al., 2006).

In the retina, cell coupling provided by GJ channels results in extensive networks (Vaney, 2002). Several Cxs have been identified in the adult rodent retina (Condorelli et al., 1998; Guldenagel et al., 2000). For example, Cx36 and Cx45 were identified as neuronal Cxs participating in the rod-mediated circuitry (Deans et al., 2002; Maxeiner et al., 2005), whereas Cx57 is apparently restricted to horizontal cells (Hombach et al., 2004).

The retina has been considered as a "natural brain slice" and largely used in studies focusing on chemical synapses. The mammalian retina contains two synaptic layers: the outer plexiform layer, which is primarily composed of ribbon synapses (Sterling and Matthews, 2005), while the inner plexiform layer largely comprises conventional synapses (Von Kriegstein et al., 1999). A considerable number of molecules have been described to participate in the chemical communication between neurons, including those that are involved in the intricate process of neurotransmitter release (Zimmermann, 1997; Parnas and Parnas, 2007). Synaptophysin is an integral membrane protein of synaptic vesicles and has an important role in the neurotransmitter release from the synaptic vesicles by making an exocytotic fusion pore (Elferink and Scheller, 1995). Synapsin I is a phosphoprotein associated with the cytoplasmic surface of the synaptic vesicle membrane and is thought to function by increasing the number of synaptic vesicles in the releasable pool (Hilfiker et al., 1999).

Based on the premise that neuronal activity may shape synaptic communication, we took advantage of the rd/rd mouse model, which in the adulthood displays natural ablation of photoreceptors (Strettoi and Pignatelli, 2000; Zhu et al., 2007) caused by a mutation in the gene encoding the β-subunit of rod cGMP-phosphodiesterase (*Pde6b*^{rd1}).

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Abbreviations: CT, cycle threshold; Cx, connexin; GJ, gap junction; PB, phosphate buffer; rd/rd, (retinal degeneration) mouse model; WT, wild-type.

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In adult rd/rd mice, nuclear staining of vertical retinal sections indicated no major abnormalities in inner layers, where only the reduction or the absence of the photoreceptor layer is evident. However, second-order neurons of the rd/rd may show morphological abnormalities, such as the atrophy of dendrites of cone bipolar cells, mostly evident at P90 (Strettoi et al., 2003). By studying this animal model, we were able to determine whether the lack of visual signaling triggered by the photoreceptors alters the expression of proteins directly involved in electrical and chemical synapses.

EXPERIMENTAL PROCEDURES

Animal procedures

Experiments were carried out with C57BL/6J (wild-type (WT)) and C3H/HeJ (rd/rd, The Jackson Laboratory, Bar Harbor, ME, USA) mice kept on a 12-h light/dark cycle (light phase 80–100 lux) with lights on at 6:00 a.m. Young adult animals (3–4 weeks old, $n=16$) were killed for different methodologies 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., Bayer, West Haven, CT, USA) between 10:00 a.m. and 12:00 a.m. All experiments were conducted in accordance to the NIH and the Institute of Biomedical Sciences/USP guidelines. Thus, we minimized the number of animals used and their suffering.

RNA isolation, cDNA synthesis and real time PCR

Retinas were directly homogenized in 1 ml TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and total RNA was extracted following the manufacturer-suggested protocol. 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 the manufacturer protocol. For each 20 μl reverse transcription reaction, 4 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. Real-Time PCR was carried out using a Rotor-Gene 6000 Real-Time Rotary Analyzer (Corbett Life Science, Sidney, Australia) with specific primers for Cx36, Cx45, Cx57, synaptophysin and synapsin I (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.

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 (ρRn) 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 15 cycles. Relative quantification of target gene expression was evaluated using the comparative CT method as previously described in detail (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 $\Delta\Delta$CT 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 $(1+E)^{-\Delta\Delta CT}$, where the E parameter corresponds to efficiency of amplification. Values were entered into a Student's t-test and P values of <0.05 were considered to be significant.

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) for 30 min and cryoprotected with a 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. For whole-mount experiments, retinas were fixed in 4% PFA in PB for 24 h at 4 °C before incubation with primary antibodies.

Retinal sections or whole-mounts were incubated overnight and for 7 days, respectively, with the primary antibodies listed in Table 1. A rabbit polyclonal antibody raised against amino acids 296–304 of human Cx36 was used (36–4600, Zymed/Invitrogen, 1:250–1:500), which identifies in the retina a single band at approximately 36 kDa (Mills et al., 2001). To identify Cx45, two different antibodies were used: a mouse monoclonal antibody (MAB3101, Chemicon, Temecula, CA, USA, 1:100–1:250) and a rabbit polyclonal antibody (AB1745, Chemicon, 1:250–1:500), 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). 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, 1:100–1:250). In Western blots, this antibody identifies the target bands at 57 and 64 kDa; the 64 kDa band likely represents a post-transcriptionally modified form of Cx57, consistent with the multiple phosphorylation sites of this protein (manufacturer information). Expression of synapsin I was investigated using a rabbit polyclonal antibody raised against a mixture of Ia and Ib isoforms purified from bovine brain (AB1543, Chemicon, 1:250). According to manufacturer information, immunolabeling is blocked by preadsorption of antibody with synapsin I. Synaptophysin expression was determined using a rabbit polyclonal antibody raised against human synaptophysin peptide coupled to ovalbumin (A0010, DakoCytomation, Copenhagen, Denmark, 1:250). Calbindin-D is a small acidic protein (28 kDa) that belongs to a family of calcium-binding proteins, and in the mouse retina is a reliable marker for horizontal cells. In some double-labeling experiments, we used a mouse monoclonal antibody raised against purified calbindin-D from chicken gut (C8666, Sigma, St. Louis, MO, USA, 1:250) in order to identify retinal horizontal cells.

After several washes, retinal sections were incubated for 2 h and whole-mounts for 24 h with goat antiserum against rabbit tagged to Alexa™ 488 (Molecular Probes, Eugene, Oregon, 1:500–1:1,000) and, in some cases, tetramethyl rhodamine isothiocyanate (TRITC, Jackson Laboratories, West Grove, PA, USA, 1:100–1:500) diluted in PB containing 0.5% Triton X-100 at room temperature. Controls for the experiments consisted of the omission of primary antibodies; no staining was observed in these cases. After washing, the tissue was mounted using Vecta Shield (Vector Laboratories, Burlingame, CA, USA).

PFA was reported to cause non-specific staining when some Cx antibodies were used (Feigenspan et al., 2001; Meier et al., 2002). Thus, in some experiments cold ethanol was used as fixative instead of PFA. Immunoreactivity pattern was very similar