UNIVERSITY OF PÉCS

Biological and Sportbiological Doctoral School

Factors influencing the expression of gap junction forming connexin proteins in the retina of vertebrate animals

PhD thesis

Tamás Kovács-Öller

Supervisor:

Béla Völgyi, PhD.
associate professor

PÉCS, 2015

1. INTRODUCTION

Vision is the most important form of sensory perception for humans. We percieve more than 75% of our environment through our vision. The base of the visual system is the eye or more specifically, it's neuronal tissue, the retina. The retina could be easily divided to inner and outer plexiform and nuclear layers, which are bordered by the vitreus from the inside and the pigment epithel from the outside.

In the vertebrate retina we can distinguish between five primal neuronal cell classes: photoreceptors, bipolar-, amacrin-, horizontal- and ganglion cells. While the photoreceptors are necessary for detecting visual information, the visual integration and transmission towards the brain is the role of the bipolar cells. The bipolar cells are excitatory, while the horizontal and amacrin cells are inhibitory. The aforementioned cell types and their projections arrange into layers, and the gap junctions are essential for forming the network between the cells (Kolb et al., 1995)

Neuronal transmissiona happens primarily through chemical synapses, but the gap junctions play an essential role as well. They serve as a molecular gateway and they can be found throughout the vertebrate body.

It is still unknown how the gap junction forming proteins, called connexins (Cx) might differ from one mammal to another, and how their expression changes during postnatal development. It is also unknown how their expression level and pattern might change in response to changes in environmental conditions. In our work, we studied the distribution of neuronal connexins in accordance with the aforementioned factors.

The different connexins were named after their molecular mass in kDa, but the base of their classificstion is sequence and functional homology (Cruciani and Mikalsen, 2006).

In the inner plexiform layer of the retina the AII amacrin cells play an important role in the retinal information transmission mainly because of their gap junctions. This is the most heavily studied amacrin cell-class in vertebrates (Kolb and Famiglietti, 1974) that has an emphasized role in the information processing of the retina.

The vision of mammals is based on a very similar neuronal network, but there can be differences differences in the number of building neurons and in the number of their connections. These differences could be explained either by the genetic distance or the mode of life of these animals.

It can be said from the overall structure of the retina that nocturnal animals have a higher number of rods (which can detect lower intensity light) than diurnal animals.

Furthermore, the retina of nocturnal animals have a reflecting layer, called the tapetum lucidum (Ollivier, 2004).

The Cx36, 45 and 57 containing gap junctions also play a role in the dynamic changes during postnatal development (Kihara et al., 2006), thus the knowledge of their expressional changes serves as starting point for understanding how the mature neuronal networks work.

2. OBJECTIVES

The gap junctions that connect the neurons are not just static channels, but also dynamic, ever-changing informration pathways. Their interoperability change is adapting to the ontogeny, and to the external and the internal dynamics of environmental factors. The goal of our research was to reveal these changes in detail. Thus we wanted to research the following:

1. The adaptation of connexin expression to evolutional trends

To this end we performed the followings:

(1) Collect the vertebrate retinal connexin aminoacid and nucleotid sequences, then create a Cx phylogenetic tree to distinguish between the Cx ortho- and paralogs.

(2) Create a Cx36 phylogenetic tree to explain the similarities between mammalian Cx36 proteins.

(3) Make Cx36 immunreactions to reveal evolutionary trends among the retinas of mammals.

2. Determine the Cx expressional changes during onthogenic development.

There is a great number of neural developmental changes in the retina in which the GJ's are actively involved. Therefore, we plan to:

(1) Examine the changes in Cx36, 45, and 57 mRNA expression in the developing retina.

(2) Describe the changes in Cx36 protein expression during early postnatal development in the rat.

(3) Characterize the developing neuronal connections that could be correlated to the expression levels of Cx36.

3. MATERIALS AND METHODS

Animals and sample preparation

Comparative studies were performed in species listed below: mouse (*Mus musculus*), rat (*Rattus norvegicus*), guinea pig (*Cavia porcellus*), rabbit (*Oryctolagus cuniculus*), hamster (*Mesocricetus auratus*), sheep (*Ovis aries*), cat (*Felis silvestris catus*), dog (*Canis lupus fam.*) ferret (*Mustela putorius furo*), squirrel monkey (*Saimiri sciureus*), swine (*Sus scrofa dom.*) and man (*Homo sapiens sapien*s). Retinal sections were made 2-4 mm from the central region in nasal direction on post-mortem material (three hours old at most). To examine the variation in the Cx ontogenic expression, P0, 1, 3, 5, 10, 15, 20 day old Wistar albino rats were used. The retinas were stored at -80 ° C. For immunocytochemistry, retinas were fixed in 4% PFA in PBS solution at 4 °C for 1 hour.

Connexin dendrogrammok

We collected the vertebrate retinal Cx gene sequences and prepared sequence homology based dendrogramms using CLC Main Workbench 6.5 software.

Polimerase chain reaction (PCR)

RNA extraction was carried out with RNazol RT from retinas stored at -80 °C. The PCR primers used in the reactions were designed specificaly to the genes of interest and were checked with NCBI Primer BLAST. As a preliminary test, total RNAs were amplified with OneStep RT-PCR with the Cx36, 45 and 57 primers. For cDNA synthesis oligo dT primers were used with the Fermentas RevertAid H- RT enzyme and protocol. The postnatal Cx36 samples were amplified with Abi StepOne Plus qPCR thermocycler and the residual samples were amplified with Bio-Rad CFX Connect. Maxima SYBR-Green was used for the reactions. The endogenous control was the Rpl13a. The data analysis was performed with the thermocycler's own software, then the data was exported and summed and normalized with Microsoft Excel. Figures were made with Origin 6.

Western blot

Retinas were homogenized in cold RIPA buffer with proteinase inhibitors. The protein concentration was determined with BCA Protein Assay Kit. The preparation of the samples was carried out according to the NuPAGE Instruction Manual. We applied 20 or 25 ug of protein per well in each run on the 10% polyacrylamide gels. The protein lanes were semi-dry blotted to PVDF membrane. After blocking, they were labeled with Cx36 rabbit polyclonal and β- tubulin rabbit polyclonal antibodies. The secondary antibodies were anti-rabbit HRP-

conjugated. After washing, ECL signal was detected using X-ray films. Exposure times were: touch, 10, 30, 60, 300 secs. The signals were digitized and were normalised to the β-tubulin signal with ImageJ (NIH) densitometry. The datapoints were adjusted to P0.

Immuncytochemistry

The 4% paraformaldehyde fixed eye cups were washed, cryoprotected and embedded. 12 micron thick sections were prepared, which were collected and fixed on gelatinized slides. The thawed sections were treated with primary antibodies (mouse anti-Cx36, rabbit anti-CaR or rabbit anti-PV) and incubated overnight. After washing and labeling with secondary antibodies, they were covered with embeding medium.

Microscopy

Sections were first photographed with 63x magnification and a DIC filter with a Nikon's FN1 type microscope. Immunlabeled sections were photographed with LSM 710 confocal laser-scanning microscope. We performed postproduction with Adobe Photoshop CS3, Illustrator CS3, and ImageJ programs. Zeiss Elyra Structured Illumination (SIM) type microscope was used to detect the high-resolution signal of Cx36. Pictures were made with Zeiss ZEN software and then analyzed with ImageJ.

To perform the densitometric and plaque statistics analysis we selected sections from the central portion of the retina with a perpendicular sectioning axis. The plaque size distributions were determined with ImageJ software's "Analyse particles" script. Statistical analysis was performed using SPSS 19 software or origin 6, and interpreted with Excel. To determine the level of significance, ANOVA analysis or Student's t-test was used.

4. RESULTS

The expression of neuronal gap junction (GJ) forming connexins is crucial for both the developing and the mature retina. Our main goal was to study the expressional changes of connexin36 (Cx36) and other retinal Cx-s as well, and to determine the factors behind the Cx expressional changes throughout evolution and the postnatal development.

Our studies resulted in the following main findings:

1.) The Cx-s developed early on during evolution and the animals in each vertebrate class inherited a set of paralog Cx-s from the common ancestor Osteichtyes animals. The conserved sequences of ortholog Cx-s attest the high importance of GJ-s in the retinal circuitry.

2.) The Cx36 proteins could be found in the retinas of all examined mammalian species and their expression patterns showed high similarity. This indicates that the functions of Cx36 GJ-s are conserved as well.

3.) The observed differences and similarities in the Cx36 plaque distribution and size correlated with life-style rather than the genetic relationship of the animals.

4.) The Cx36 mRNA expression level increased during postnatal development until it reached a peak at P15, which is highly related to the neuronal reorganization around eye opening and the formation of the rod signaling system.

5.) The Cx45 and Cx57 transcripts also display characteristic expressional changes during the early postnatal development, which clearly correlates with the formation and maturation of the GJ-s they comprise.

Our findings show evidence that retinal GJ-s serve fundamental functions that only allow slow, conservative changes in the Cx sequences throughout evolution. On the other hand, retinal GJ-s are dynamic, reconfigurable inter-neuronal connections, whose quantity, molecular makeup and conductance adapts to the ever-changing environment. In accordance with the latter statement, this study presented faster and slower developmental changes in the Cx36 expression level.

PUBLICATIONS

PEER-REVIEWED ARTICLES INTHE TOPIC OF THE THESIS

Kovács-Öller T, Raics K, Orbán J, Nyitrai M, Völgyi B. (2014) Developmental changes in the expression level of connexin36 in the rat retina. Cell Tissue Res., 358(2):289-302. *IF 3.33*

Völgyi B, Kovács-Öller T, Atlasz T, Wilhelm M, Gábriel R. (2013) Gap junctional coupling in the vertebrate retina: variations on one theme? Prog Retin Eye Res. 34:1-18. *IF 9.90*

ORAL AND POSTER PRESENTATIONS INTHE TOPIC OF THE THESIS

Debertin G and Kovács-Öller T, Balogh M, Popovich E, Orbán J, Lukáts Á, Énzsöly A, Balogh L, Kántor O, Völgyi B. (2014) Multiple species comparison of the gap junction forming Connexin36 in the mammalian retina. 1st Innovation in Science 2014, Doctoral Student Conference, Szeged, Magyarország

Kovács-Öller T, Balogh M, Raics K, Nyitrai M, Völgyi B. (2013) Developmental changes in the gap-junction forming connexin36 expression levels in the rat retina. European Retina Meeting 2013, Alicante, Spanyolország

Kovács-Öller T, Balogh M, Pénzes M, Orbán J, Lukáts Á, Énzsöly A, Balogh L, Kántor O, Völgyi B. (2013) Dispersion of retinal Connexin36 in mammalian carnivores. II. Interdiszciplináris Doktorandusz Konferencia, Pécs, Magyarország

Balogh M, Pénzes M, Kovács-Öller T, Lukáts Á, Énzsöly A, Balogh L, Kántor O, Völgyi B. (2013) Distribution of connexin36 in the retina of various vertebrate species. János Szentágothai Memorial Conference and Student Competition, Pécs, Magyarország

Kovács-Öller T, Völgyi B. (2013) Developmental changes in the gap-junction forming connexin36 expression levels in rat retina. MITT 2013, Budapest, Magyarország

Kovács-Öller T, Popovich E, Völgyi B. (2012) Genetic relations of vertebrate retinal connexins. János Szentágothai Memorial Conference and Student Competition, Pécs, Magyarország

OTHER PEER-REVIEWED ARTICLES

Debertin G, Kántor O, Kovács-Öller T, Balogh L, Szabó-Meleg E. (2015) Tyrosine hydroxylase positive perisomatic rings are formed around various amacrine cell types in the mammalian retina. J Neurochem.,134(3):416-28.

Kántor O, Varga A, Tóth R, Énzsöly A, Pálfi E. (2015) Stratified organization and disorganization of inner plexiform layer revealed by TNAP activity in healthy and diabetic rat retina. Cell Tissue Res., 359(2):409-21.

Kántor O, Varga A, Kovács-Öller T, Énzsöly A, Balogh L. (2014) TNAP activity is localized at critical sites of retinal neurotransmission across various vertebrate species. Cell Tissue Res., 358(1):85-98.

Völgyi B, Debertin G, Balogh M, Popovich E, Kovács-Öller T. (2014) Compartment-specific tyrosine hydroxylase-positive innervation to AII amacrine cells in the rabbit retina. Neuroscience. 270:88-97.

Jakab F, Sebok J, Szántó Z, Hang D, Imre M. (2011) Dobrava-Belgrade hantavirus infection mimics acute appendicitis. J Clin Virol.,50(2):164-6.

OTHER ORAL AND POSTER PRESENTATIONS

Debertin G, Kántor O, Kovács-Öller T, Balogh L, Szabó-Meleg E, Orbán J, Nyitrai M, Völgyi B. (2015) Tyrosine hidroxylase positive perysomatic rings are action sites for both dopamine and GABA to target mixed population of amacrine cells. ERM 2015, Brighton, Nagy-Britannia

Debertin G, Kovács-Öller T, Popovich E, Orbán J, Nyitrai M, Völgyi B. (2014) Dopaminergic amacrine cell dendrites form presynaptic rings around somata of various inner retinal interneurons in the mammalian retina. III. Interdisciplinary Doctoral Conference, Pécs, Magyarország

Balogh M, Kovács-Öller T, DebertinG, Völgyi B. (2014) Observation of the correlated ganglion cell activity in the mouse retina by using Ca++ -imaging. IBRO Workshop 2014 (P105.), Debrecen, Magyarország

Kovács-Öller T. (2014) A retina egy neurobiológus szemén keresztül. Kutatók éjszakája PTE-SZKK, Pécs, Magyarország

Kántor O, Varga A, Kovács-Öller T, Énzsöly A, Balogh L, Négyessy L, Völgyi B, Lukáts Á. (2014) TNAP activity is localized in critical sites of retinal neurotransmission across various vertebrate species. IBRO Workshop, Debrecen, Magyarország; P38.

Balogh M, Kovács-Öller T, DebertinG, Völgyi B. (2013) Detection of correlated gamglion cell activity in the mammalian retina by using Ca++-imaging technique, 10th János Szentágothai Transdisciplinary Conference and Student Competition, Pécs, Magyarország

Knapp L, Kocsis K, Kovács-Öller T, Gellért L, Varga D, Ozsvár A, Kis Z, Völgyi B, Toldi J and Farkas T. (2013) Mechanisms involved in kainic acid-induced postconditioning after global ischemia in the rat hippocampus. 7th International Symposium on Experimental and Clinical Neurobiology, Kassa, Szlovákia

Kocsis K, Knapp L, Kovacs-Öller T, Gellert L, Olah G, Heredi J, Menyhart A, Kis Zs, Völgyi B, Toldi J, Farkas T. (2013) Combination of oxaloacetate and acetyl-L-carnitine posttreatment prevents functional impairment in the hippocampus after global hypoperfusion.(SNN-6). 7th International Symposium on Experimental and Clinical Neurobiology, Kassa, Szlovákia

Kocsis K, Knapp L, Kovács-Öller T, Berkó A, Pósa A, Kis Zs, Varga Cs, Völgyi B, Farkas T, Toldi J. (2013) Mechanisms involved in kainate-induced postconditioning in ischemic hippocampus. MITT 2013, Budapest, Magyarország

Németh V, Madai M, Maráczi A, Bérczi B, Horváth Gy, Oldal M, Kovács T, Bányai K, Jakab F. (2011) Dobrava-Belgrade hantavírusok gyakoriságának vizsgálata rágcsálókban magyarországi és észak-horvátországi területeken. PTE Innovációs Nap 2011, Pécs, Magyarország

Németh V, Madai M, Maráczi A, Bérczi B, Horváth Gy, Oldal M, Kovács T, Bányai K, Jakab F. (2011) Seroprevalence and genetic characterization of Dobrava and Saaremaa hantaviruses among rodents (Apodemus agrarius, Apodemus flavicollis) in Hungary and Northern Croatia. International Meeting on Emerging Diseases and Surveillance (IMED) 2011, Vienna, Austria

Németh V, Madai M, Bérczi B, Maráczi A, Horváth G, Oldal M, Kovács T, Jakab F. (2010) Seroprevalence and genetic characterization of Dobrava and Saaremaa hantaviruses among rodents (Apodemus agrarius, A. flavicollis) in Hungary and Northern Croatia. 20th ECCMID Vienna, Austria, p.137:P1396

Németh V, Madai M, Maráczi A, Bérczi B, Horváth Gy, Oldal M, Kovács T, Bányai K, Jakab F. (2010) Prevalence of Dobrava-Belgrade and Saaremaa Hantaviruses among Apodemus mice in Hungary and Northern Croatia. A Magyar Mikrobiológiai Társaság 2010. évi Nagygyűlése, Keszthely, Magyarország. p. 35.;VE-20.

SCIENTOMETRICS

CUMMULATIVE IMPACT FACTOR: 30,93

CITATIONS: 27

INDEPENDENT CITATIONS: 19