Doctoral (Ph.D.) Thesis

GABA signaling in developing mouse lens

Andrea Kwakowsky

Supervisor: Dr. Gábor Szabó, MD, Ph.D.

Department of Gene Technology and Developmental Neurobiology
Laboratory of Molecular Biology and Genetics
Institute of Experimental Medicine
of the Hungarian Academy of Science

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Introduction

γ-aminobutyric acid (GABA), the principal inhibitory neurotransmitter in the central nervous system (CNS), during development appears long before the onset of synaptogenesis and acts as an epigenetic factor to control processes including cell proliferation, migration and dendritic maturation. GABA is also expressed from very early embryonic stages at multiple sites outside of the CNS, where it may serve as a trophic factor regulating development. GABA effects appear to be mediated through a paracrine, diffuse, non-synaptic mode. During the past few years, enormous progress has been made in understanding the role of GABA during development. Due to the diversity of GABA action and the complexity of the GABA signaling system, the molecular mechanisms underlying it, however remain largely unknown.

Because of its simplicity and its predictable pattern of development and differentiation, the lens has attracted the attention as a model system to study the developmental role of different signaling molecules. In contrast to the cellular and molecular complexities present in most other tissues, particularly in the CNS, the lens is a relatively simple system, composed of a single layer of metabolically active epithelial cells that differentiate into quiescent, but structurally highly differentiated fiber cells. Furthermore, the vertebrate lens provides an ideal model for studying complex signaling pathways operating during embryonic development afforded by the regional compartmentalization of cell proliferation (lens epithelial cells) and differentiation (fiber cells). To this end, lens could be an ideal model system to uncover the general mechanism of GABA action in key developmental processes such as proliferation and differentiation.

This thesis summarizes the results of a series of experiments aimed at elucidating the developmental role of GABA and the molecular mechanism of its signaling using a simple model system, the developing mouse lens.
Specific aims

The major objective of the research was to characterize and dissect the role of GABA signaling in development and to uncover the underlying mechanisms using mouse lens as a model system. The specific aims followed in the course of this work could be summarized as follows:

1. Detection and characterization the temporal and spatial expression of different GAD forms and GABA in the mouse lens during development.
2. Identification and characterization the expression of GABA signaling components in the developing mouse lens: the GABA<sub>A</sub> and GABA<sub>B</sub> receptor subunits, vesicular and membrane GABA transporters.
3. Establishment and characterization of primary mouse lens epithelial cell culture to study GABA signaling in the lens in vitro.
4. Testing the functionality of GABA signaling in intact lens and in epithelial cell culture by calcium-imaging using confocal laser microscopy.
5. Generation and characterization of transgenic mice that overexpress GAD67 in the lens under the control of lens specific αA-crystalline promoter.
6. Determining the effects of altered GABA levels on proliferation and differentiation of the developing lens using CrysGAD67 transgenic mice and GAD65/GAD67 knock out mice.

Methods

1. Animals

For the studies we have used C57Bl6 (Charles Rivers, Hungary), FVB/Ant (Errijgers et al., 2007) wild-type mice, GAD65-GFP, CrysGAD67 transgenic mice as well as GAD65 and GAD67 single and double knock-out (KO) mice, which were housed in the SPF animal facility of the Institute of Experimental Medicine.

2. Genetically modified mouse lines used in this study

GAD65-GFP transgenic mouse line used in this study contains 5.5 kb of the GAD65 promoter fused to eGFP in the third exon of GAD65. GAD67-GFP knock-
in/GAD65 knock-out mouse line was obtained from Yuchio Yanagawa. CrysGAD67 transgenic mice were generated within the scope of the study outlined here.

### 3. Generation of transgenic mice

To make the Crys-GAD67-GFP transgenic construct, GAD67-hCGFP fusion gene was inserted into αA-crystallin promoter vector CPV-2 between the promoter and the splicing/polyadenylation signals from the early region of SV40 virus. Transgenic mice were produced by injecting isolated DNA fragment into male pronuclei of fertilized oocytes of inbred FVB/N mice. Potential transgenic founder pups were identified by Southern hybridization using P32-labeled SV40 fragment as a transgene specific probe. Progenies were genotyped by transgene specific PCR using tail DNA. CrysGAD67 homozygous mice were selected by Dot blot analysis and were backcrossed to wild type mice to prove homozygous status for the transgene.

### 4. Semi-quantitative RT-PCR amplification

Lenses were collected and total RNA was isolated from tissue. The concentration and purity of the RNA preparations were determined by measuring optical density at 260 and 280 nm for all preparations. Three micrograms of RNA were reverse-transcribed using RevertAid™ H Minus First Strand cDNA Synthesis Kit with random hexamer primers. PCR amplification was performed on one tenth of the first strand cDNA by using gene or transgene-specific primers. For quantification of expression, β-actin was co-amplified with the target gene for 20 cycles.

### 5. Western blotting

Lens protein extracts were prepared by homogenizing freshly isolated lenses in SB buffer. 30 µg of protein samples were run on 10% SDS-PAGE, blotted. Then membranes were reacted with primary antibody followed by appropriate alkaline phosphatase conjugated secondary antibody and developed. GAD forms were identified as 30-31 (corresponding to GAD25), 44, 65 and 67 kDa bands, respectively, using anti-pan GAD rabbit serum # 6799.
6. Immunohistochemistry and immunocytochemistry

Tissues and primary lens epithelial cell cultures were fixed in 4% (w/v) paraformaldehyde (PFA) in PBS, and in 4% PFA-0.1% glutaraldehyde-PBS for GABA detection. The tissue was sectioned at 20 or 25 µm on a cryostat.

Sections and primary epithelial cell cultures were incubated with primary antibody (rabbit anti-GAD serum #6799, rabbit anti-GAD65 serum N65, rabbit anti-GABA, rabbit anti-DLX2, rabbit anti-GABA_Aβ3, rabbit anti-GABA_B2, guinea pig anti-VGAT, guinea pig anti-mouse GAT1, rabbit anti-GAT3, rabbit anti-Ki-67, mouse anti-N-cadherin, mouse anti-Pan-cadherin, goat anti-αA-crystallin and rabbit anti-αB-crystallin) followed by the appropriate secondary antibody. The reaction was visualized by one of the followings: Streptavidin-conjugated Cy3, Streptavidin-conjugated Oregon Green-488 and ExtrAvidin-HRP reacted with diaminobenzidine in the presence H2O2. In some cases we used secondary antibodies directly conjugated to fluorescent dyes (anti-mouse (guinea pig)-Alexa Fluor® 594, anti-goat (anti-rabbit, anti-mouse)-Alexa Fluor® 488). Specimens were examined under Zeiss Axioscop-2 microscope or confocal laser scanning microscope Zeiss LSM 510 META and Olympus FV500.

7. Non-radioactive in situ hybridization

GAD67 antisense riboprobe was used to detect GAD67 mRNA in wild type lenses. In CryxGAD67 lenses transgene derived mRNA was detected by using SV40 riboprobe. Sense probes were used as controls for in situ hybridization. DIG-α-UTP was used to generate non-radioactive dioxigenin labeled RNA probes. After hybridization and washings human placental alkaline phosphatase conjugated anti-DIG antibody was applied to detect probes bound to corresponding RNA. Hybridization was visualized by alkaline phosphatase enzyme reaction.

8. GABA measurement by high performance liquid chromatography

Frozen lens tissue was homogenized in 0.2 M ice-cold perchloric acid, cleared by centrifugation and neutralized. Derivatized 1-(alkylthio)-2-alkylisoindol amino acid adducts were separated on a 5 µm Discovery HS C-18 (150x4.6 mm) analytical column. Amino acid derivatives were detected by filter fluorometer at 340 nm excitation and 455-
nm emission wavelengths. Concentrations were calculated by a two-point calibration curve internal standard method.

9. Primary lens epithelial cell culture

Mouse P0 lenses were dissected from deeply anaesthetized newborn (P0) mice in ice-cold PBS, subsequently transferred into 0.02% EDTA solution in 0.5 mM DPBS at 37°C for 10 min. Cells were then centrifuged and resuspended in fresh Medium 199-10% fetal calf serum and plated on matrigel-coated coverslips. Two week-old cultures were used for immunocytochemistry and Ca^{2+} imaging.

10. [Ca^{2+}]_i imaging studies

Freshly isolated lenses and primary lens epithelial cell cultures from neonatal mice were loaded with Fluo-4/AM calcium sensitive dye and superfused with GABA or GABA receptor agonists and antagonists. Ca-imaging analysis on intact lenses was performed using a laser-scanning microscope, LSM, Olympus FV-500 and the imaging software, FluoView™. Primary lens epithelial cell cultures were observed under Zeiss LSM 510 META laser-scanning microscope. Analyses were performed at the single-cell level and expressed as relative fluorescence intensity.

11. Statistical analysis

Quantitative data are presented either as representative single experiments, or the mean ± S.E.M. based on pooled data from several experiments. Significant differences among groups were evaluated by a Student-test (Ca^{2+} imaging, cell proliferation data). A value of *p<0.05 was accepted as an indication of statistical significance. RT-PCR data were analyzed with Spearman correlation test using GrapPad Prism 5.0 software. In all cases, p<0.05 was considered significant.
Results and Conclusions

1. GAD isoforms exhibit distinct spatiotemporal expression patterns in the developing mouse lens: correlation with Dlx2 and Dlx5

- We showed for first time that γ-aminobutyric acid (GABA) and all forms of its biosynthetic enzyme GAD (EGAD, GAD65, GAD67) are expressed in the mouse lens from early developmental stages.
- Our results demonstrate that the GAD forms display developmental stage-specific expression: GAD65 and EGAD predominating during primary fiber differentiation, and GAD67 being most abundant in the postnatal secondary fiber cells.
- GABA can be detected from the earliest stage of the lens development and peaks during the most extensive secondary fiber cell differentiation and elongation.
- GABA and GAD are most abundant at the tips of elongating fibers and are absent from organelle-free cells, suggesting their involvement is primarily in shaping of the cytoskeleton during fiber elongation stages.
- We demonstrated that Dlx2 and Dlx5 transcription factors, known as upstream regulators of GAD, are expressed in an sequential manner in the developing lens. Dlx2 expression was induced before Dlx5. The temporal expression of Dlx2 parallels that of EGAD and GAD65, suggesting that Dlx2 may be needed for induction and/or maintenance of the “early” GAD forms. Dlx5 expression correlates only partially with that of GAD67: its initial up-regulation precedes that of GAD67 but unlike GAD67, Dlx5 is not expressed beyond P14 suggesting that Dlx5 may be needed for induction, but not for maintenance of GAD67 expression.

2. GABA neurotransmitter signaling in the developing mouse lens: dynamic regulation of components and functionality

- We also detected different GABA_A and GABA_B receptor subunits, GABA transporters (vesicular VGAT and membrane GAT) in the developing mouse lens as well as in primary epithelial lens cultures (LEC), and characterized their temporal and spatial expression during development.

GABA_A-R, GABA_B-R and VGAT were greatly enriched in the apical/basal
membranes of both lens epithelium and fiber cells showing especially prominent staining in the posterior sutures. In the lens VGAT was more abundant before birth and in comparison with GATs, it was never detected on the lateral surfaces. The specific high accumulation of VGAT in lens cells, suggests that it may have a role for in vesicular GABA transport.

Furthermore, different GATs are selectively sorted to the apical or basolateral membranes where they may actively participate in the ion exchange, regulation of the ion homeostasis and cell volume. In the lens epithelium GAT1 and GAT3 showed similar, predominantly apical/basal localization, but their patterns in the fibers differed significantly. GAT1 was enriched in both apical/basal and lateral membranes, while GAT3 showed a unique pattern, being localized in apical and lateral membranes, but not in the basal tips of fibers forming the posterior suture.

We found, that different components of a functional GABA signaling system are expressed in a strict spatio-temporal manner correlating with different phases of lens development. Our data indicate a clear switch from embryonic to postnatal expression of different components. In the lens, the GABA_{A}R (and probably GABA_{B}R) subunit switch strongly correlates with the switch from mainly apo-GAD65 and EGAD to the constitutively active GAD67 and gradual substitution of VGAT with membrane GABA transporters (GATs). Our data strongly suggest that in the embryonic lens GABA synthesized by GAD65 is released predominantly by VGAT and binds to GABA_{A}R \alpha_{2,3} subunit-containing receptors, while GABA produced by GAD67 is released predominantly by the membrane GAT2 and binds to \alpha_{1}-containing GABA_{A}R. GABA made by the enzymatically active GAD44, which is co-expressed with GAD65 and is the embryonic counterpart of GAD67, may be released by inverse operation of one or more membrane GATs.

- We showed that both GABA_{A} and GABA_{B} receptor activation evoked transient increase of [Ca^{2+}]_{i} in intact lenses and in primary LEC, demonstrating the presence of functional GABA signaling in the developing lens.

Since we demonstrated that cells located in the germinative and migratory zones of the lens epithelium and elongating primary or secondary fibers express a functional GABA signaling system, GABA may act in a paracrine/autocrine fashion to modulate the
cell cycle, cell migration and fiber elongation through modulation of the \([\text{Ca}^{2+}]_i\) level, by analogy with its similar role in the ventricular and subventricular zones of the embryonic nervous system.

3. **In vivo role of GABA in multiple steps of lens development as revealed by studies on mouse models with genetically altered GAD expression**

- To evaluate the role of GABA during lens development *in vivo*, we generated and characterized a transgenic mouse model overexpressing GAD67 in the lens, and also studied the embryonic lens phenotype of GAD65 and GAD67 knock-out mice.

- Mice with elevated GAD67 and GABA levels in the lens showed accelerated elongation of primary fiber cells at lens vesicle stage, while the same process was delayed in mice lacking GAD65, but not in GAD67 \(-/-\) mutant, indicating that GABA synthesized by GAD65 plays a role in regulating primary fiber cell elongation.

- Cell proliferation tests showed increased epithelial cell proliferation in the germinative zone in mice overexpressing GAD67 and decreased proliferation in mice lacking GAD67, but not in GAD65/- mutant. These findings indicate that GABA produced by the adult GAD67 has a role in late epithelial cell proliferation that is a source for secondary fiber cell generation.

- Late embryonic and adult lenses with elevated GAD67/GABA levels displayed multiple defects including deformed fiber tips and open lens suture, disorganized fiber cells and cataract, all are consistent with the role for GABA in differentiation and structural organization of secondary fiber cells.

In summary, our results indicate that GABA is involved in trophic signaling from early stages of the lens development, probably as a modulator of proliferation, fiber cell differentiation and migration, similar to that operating during embryonic and adult neurogenesis. Based on expression analysis of GABA signaling components and the phenotype of transgenic mice with altered GABA level, we suggest that GABA synthesized by early GADs may play a role in primary fiber cell differentiation. While, GABA produced by GAD67 may regulate the cell proliferation in epithelial germinative zone and the differentiation of secondary fiber cells. GADs through GABA have also
been involved in the regulation of cell adhesion, a process that is a key component in determining lens structure.

List of Publications

I. Full papers:


3. Andrea Kwakowsky, Marija Schwirtlich, Zsuzsa Emri, Zoya D. Katarova and Gábor Szabó. “GABA_A and GABA_B receptor-induced Ca transients in primary lens epithelial cell cultures expressing active GABA signaling components.” Exp. Eye Res. (under submission)

II. Poster presentations:

2. Z. Katarova, A. Kvakovszki, M. Schwirtlich, F. Erdélyi, I. Szatmári and G. Szabó. „Role of GABA signaling in the developing eye: ocular defects caused by overexpression of different GAD forms in the lens of transgenic mice.” MITT IX. 2003, Balatonfüred


4. Zoya Katarova, Andrea Kvakovszki, Marija Schwirtlich, Marija Baranyi and Gábor Szabó. „GAD expression is critical for eye development at late embryonic stages.” JAX Neurogenetics Conference V. 2004, BarHarbor


III. Oral presentations:
