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GABA signaling in developing mouse lens

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Abbreviations used:

AAH	artificial aqueous humor
ACh	acetylcholine
AET	aminoethylisothiouronium bromide
Bac	baclofen
Bic	bicuculline
BPH	benign prostatic hypertropy
BrdU	5-bromodeoxyuridine
BSA	bovine seum albumin
C	cornea
$[Ca^{2+}]_i$	intracellular calcium concentration
CGP	CGP55845
CaM	calmodulin
Chat	cholineacetyltransferase
$[Cl^-]_i$	intracellular chloride concentration
CNS	central nervous system
CP	cortical plate
DAB	diaminobenzidine
DABCO	1,4-diazabicyclo-[2,2,2]-octane
DAPI	4',6-diamidino-2-phenylindole
DEPC	diethyl pyrocarbonate
DIG	dioxigenin
Dlx	Distal-less homeobox genes
DNA	deoxynucleic acid
EDTA	ethylendiaminetetraacetic acid
E_{GABA}	GABA receptor equilibrium potential
EGAD	embrionic GAD
EGF	epidermal growth factor
EGTA	ethylene glycol tetraacetic acid
ES cells	embryonic stem cells
EQ	equatorial region, equator
FBS	fetal bovine serum
FCS	fetal calf serum
FGF	fibroblast growth factor

GABA	γ-aminobutyric acid
$GABA_A$	GABA A receptor
$GABA_B$	GABA B receptor
$GABA_C$	GABA C receptor
$GABA_BR1$	GABA B receptor R1 subunit
$GABA_BR2$	GABA B receptor R2 subunit
GABAR	GABA receptor
GABA-T	GABA-transaminase
GABRP	GABA A receptor π subunit
Gcl	ganglion cell layer of retina
GAD	glutamic acid decarboxylase
GAT	GABA transporter
GFP	green fluorescent protein
GnRH	gonadotropin-releasing hormone
HBSS	Hepes-buffered saline solution
HGF	hepatocyte growth factor
^{3}H-thymidine	tritiated-thymidine
IDDM	insulin-dependent diabetes mellitus
IGF	insulin-like growth factor
Inbl	inner neuroblastic layer of retina
Ipl	inner plexiform layer of retina
IPSP	inhibitory postsynaptic potential
IZ	intermediate zone
KCC	kalium-chloride co-transporter
KI	knock-in
KO	knock-out
L	lens
LC	lens cup
LE	lens epithelium
LEC	lens epithelial cell cultures
LF	lens fibers
LHRH	luteinizing hormone-releasing hormone
LN	lens nucleus
LP	lens placode
LSM	laser-scanning microscope

LV	lens vesicle
M	muscimol
MIP	major intrinsic protein
MMP	matrix metalloproteinase
MW	microwave oven
mRNA	messenger ribonucleic acid
NKCC	sodium potassium chloride co-transporter
NR	neural retina
OV	optic vesicle
P0	postnatal day 0
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PDAC	pancreatic ductal adenocarcinoma
PDGF	platelet-derived growth factor
Pf	primary fiber cells
PFA	paraformaldehyde
PLE	presumptive lens ectoderm
PLP	pyridoxal 5'-phosphate
PMSF	phenylmethylsulfonyl fluoride
R	retina
RT	room temperature
RT-PCR	reverse-transcriptase polymerase chain reaction
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SSA	succinic aldehyde
SSADH	succinic semialdehyde dehydrogenase
SSC	sodium chloride sodium citrate buffer
SVZ	subventricular zone
TBS	Tris-buffered saline
TCA cycle	tricarboxylic-acid cycle
TGF	transforming growth factor
VGAT	vesicular GABA transporter
VGCC	voltage-gated calcium channel
VZ	ventricular zone

I. Introduction

During development, multicellular organisms must coordinate the growth, differentiation, and maintenance of many different cell types. To achieve this coordination, each cell must continuously integrate a complex array of signals, including both inductive and inhibitory cues, and then translate these instructions into spatially and temporally appropriate developmental responses.

In the last fifty years, a large number of secreted molecules have been identified and shown to significantly influence individual developmental programmes. Neurotransmitters are a class of secreted molecules that might be important signals in the regulation of development. During brain development, neurotransmitters are expressed before synapse formation, suggesting that their action is not restricted to synaptic transmission. This developmental role may be related to and evolved from their ancient function in lower organisms where these substances act as both intra- and intercellular signaling devices. In the past few decades we have seen tremendous progress in deciphering the early signaling role of neurotransmitters.

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. These 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. Recent data have shown that all components of the GABA signaling system are expressed from very early embryonic stages, not only in the CNS, but also in non-neuronal structures.

Due to the diversity of GABA action and the complexity of the GABA signaling system, the molecular mechanisms underlying it, however remain unknown. Inactivating of a number of genes of this pathway in mice has not brought us closer to fully understand GABA action and the underlaying signaling mechanisms. This dissertation summarizes the results of a series of experiments aimed to elucidate the developmental role of GABA and the molecular mechanism of its signaling using a simple model system, the developing mouse lens.

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 GABA signaling. 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).

We described for first time the presence and dynamic temporal and spatial regulation of all molecular components of GABA signaling in the developing mouse lens. Furthermore we showed that these components are fully active as GABA or specific GABA-R agonists evoked GABA receptor activation, transient increase of intracellular calcium concentration ($[Ca^{2+}]_i$) in intact newborn mouse lens and in primary lens epithelial cell cultures (LEC). In addition we used an *in vivo* molecular genetic approach, which allows us to study the role of GABA signaling in a highly selective area of the intact organism without interfering with functions that are crucial for its survival. We have created transgenic mice overexpressing the 67 KDa form of glutamic acid decarboxylase (GAD67), the enzyme catalyzing the rate limiting step in the production of GABA, under the regulation of the αA-crystallin promoter, which drives lens specific expression. We also analyzed lenses of mice lacking both GAD isoforms. Detailed phenotypic and molecular analysis of genetically modified mouse models with altered GABA levels helped us to understand the molecular mechanism of GABA action in development. Our results show that GAD and GABA are involved in the epithelial cell proliferation, fiber cell differentiation and cell adhesion during lens development by modulating the $[Ca^{2+}]_i$ levels.

The results of these studies will have an impact not only on understanding the complex mechanisms directing normal ocular development but also will help to design new therapies to prevent loss of vision in many diseases in which the lens function might be affected by the altered GABA signaling in the lens such as cataract. Understanding GABA signaling in the lens may also provide us with tools to uncover the involvement of GABA in brain development and in developmental disorders.

The experimental work, part of which is included in this dissertation was performed at the Department of Gene Technology and Developmental Neurobiology, Laboratory of Molecular Biology and Genetics, Institute of Experimental Medicine, under supervision of Dr. Gábor Szabó and Dr. Zoya D. Katarova.

II. Background and significance

1. Diverse function of GABA from bacteria to man: GABA is more than an inhibitory neurotransmitter

GABA is a ubiquitous four-carbon, nonprotein amino acid that is conserved from bacteria through yeast to vertebrates. GABA had been long known to exist in bacteria and plants, where it serves a metabolic role in the Krebs cycle (Work and Dewey, 1953; Marcus and Halpern, 1969; Bouche and Fromm, 2004). In both bacteria and yeast, GABA uptake and biosynthesis are mainly involved in nitrogen and carbon metabolism (Shaibe et al., 1985; Kumar and Punekar, 1997; Solomon and Oliver, 2002), although other functions such as GABA synthesis for pH regulation in *Escherichia coli* and for normal oxidative stress tolerance in *Saccharomyces cerevisiae* have also been postulated (Coleman, 2001; Yohannes, 2004). In plants, rapid GABA accumulation occurs in response to a variety of stress (Bown and Shelp, 1997; Shelp et al., 1999; Kinnersley and Turano, 2000). Several papers demonstrate that plant-derived extracellular GABA mediates communications between plants and animals, fungi, bacteria, and other plants (Shelp et al., 2006). Recent evidence indicates a new possible role of GABA as a signal molecule in *Arabidopsis thaliana*, regulating processes like the growth and guidance of pollen tube to the female gametophyte (Bouche et al., 2003).

While GABA is now established as the principal inhibitory neurotransmitter in the mammalian CNS, its full acceptance in this role happened only relatively recently. In 1950, Eugene Roberts and Jorge Awapara independently discovered that there were prodigious amounts of GABA in the mammalian central nervous system (1mg/g). During the 1950 and 1960s, strong evidence accumulated that GABA may act as an inhibitory neurotransmitter in both vertebrate and invertebrate nervous systems. Establishing its role as a transmitter was a lengthy process, GABA was not accepted as neurotransmitter until the 1960's after a great deal of physiological experimentation (Florey and McLennan, 1955; Florey,1991; Bazemore et al., 1957; Roberts, 1962; Delcastillo etal., 1963; Del Castillo etal., 1964). Over the past half-century, studies have lead to very important discoveries, but the functional role of GABA and its mechanism of action are still far from being fully understood.

In vertebrates at least 30% of synapses in the brain are GABAergic (Docherty et al., 1985). GABA is involved in almost all brain functions such as locomotor activity, memory, learning, reproduction and circadian rhythm (Agmo et al., 1987; Paulsen and Moser, 1998; Mintz et al., 2002; Leventhal et al., 2003; Terasawa, 2005).

Outside the CNS GABA is also involved in processes, such as the control of hormone

release in endocrine cells in the pancreas, adrenal medulla and the gastrointestinal tract in animals (Tillakaratne et al., 1995). The GABA innervation appears to play a role in patterning the pulsatile discharge of oxytocin cells that is observed both during parturition and during suckling-induced reflex milk ejection (Moos, 1995; Voisin et al., 1995). Furthermore, GABA stimulates oxidative stress tolerance and hyaluronic acid production from dermal fibroblasts (Ito et al., 2007).

In addition during embryonic development GABA has been proposed to serve as a trophic factor for differentiating neurons and some nonneuronal cells also, regulating key developmental steps such as proliferation, differentiation and migration (Lipton and Kater, 1989; Lauder, 1993; Owens and Kriegstein, 2002).

Altered GABA-mediated signaling is likely to play a role in a number of pathological conditions affecting the nervous system such as Parkinson's disease, epilepsy, stiff-person syndrome, schizophrenia, depression, anxiety and panic disorders (Wong et al., 2003).

All these findings indicate that GABA has a diverse role in living organisms and its function becames more complex through evolution and changes within one organ during development.

2. GABA signaling and its molecular components

In the mammalian brain, γ-aminobutyric acid is converted primarly from glutamic acid by the action of glutamic acid decarboxylase (GAD). GABA is transported into synaptic vesicles by vesicular GABA transporter (VGAT) (Fon and Edwards, 2001) and is released from nerve terminals vesicularly upon stimulation. However, non-vesicular forms of GABA secretion have also been described. It can also be released by the reversal of membrane GABA transporters (GATs) (Taylor and Gordon-Weeks, 1991) (Fig. 1).

GABA mediates its effects through three receptor types, the ionotropic $GABA_A$ and $GABA_C$ and metabotropic $GABA_B$ receptors. GABA signals are terminated by rapid re-uptake via membrane GABA transporters. GATs are specific high-affinity, Na(+)/Cl(-) dependent transporters, which regulate extracellular levels of GABA by reuptake of the neurotransmitter into nerve terminals and into surrounding glial cells, where GABA is metabolized by a transamination reaction that is catalysed by GABA transaminase, a mitochondrial enzyme (Baxter, 1976) (Fig. 1).

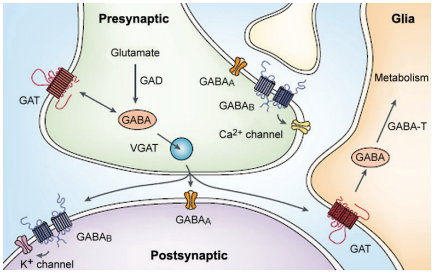

Fig. 1. Components of the GABA signaling pathway. Schematic diagram of the synthesis and transport of GABA at synapses. GABA is synthesized primarily from glutamate in a reaction that is catalysed by GAD. GABA can be released either vesicularly (by VGAT) or nonvesicularly (by reverse transport). GABA receptors are located at pre- and postsynaptic sites. Reuptake of GABA by surrounding neurons and glia occurs through the activity of GABA transporters (GAT). GABA is metabolized by GABA transaminase (GABA-T). Modified from Ref.: *Owens and Kriegstein, 2002-Nature Neuroscience*

3. Regulation of GABA synthesis: GADs

3. 1. The synthetic enzyme for GABA-glutamic acid decarboxylase (GAD)

Regulation of GABA-mediated signaling involves several mechanisms, among which modulation of GABA synthesis by the rate-limiting enzyme glutamate decarboxylase (GAD) plays a central role. Glutamate decarboxylase is a pyridoxal 5'-phosphate (PLP)-dependent enzyme, which catalyses the irreversible α-decarboxylation of L-glutamate to γ-aminobutyric acid (Fig. 2).

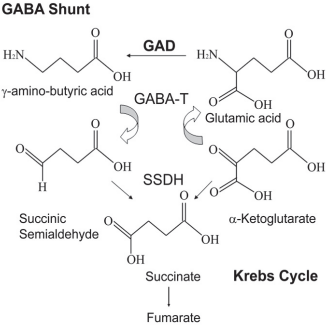

Fig. 2. Schematic representation of the GABA shunt metabolic pathway. The GABA shunt is composed of three enzymes: GAD, GABA-T and SSADH. The GAD is a cytosolic enzyme that catalyzes the irreversible decarboxylation of glutamate to produce GABA. GABA can be metabolized by a transamination reaction with α-ketoglutarate, catalized by GABA-T. Succinic aldehyde (SSA) is reduced by a SSADH to form succinate, which enters the TCA cycle.

GABA is mainly metabolized through a short pathway called the GABA shunt, because it bypasses two steps of the tricarboxylic-acid (TCA) cycle. The pathway is composed of three enzymes: the cytosolic glutamate decarboxylase (GAD) and the mitochondrial enzymes, GABA transaminase (GABA-T) and succinic semialdehyde dehydrogenase (SSADH) (Fig. 2).

GAD is widely distributed amongst eukaryotes and prokaryotes, but its function varies in different organisms. In *Escherichia coli*, this enzyme is expressed in response to environmental stresses to maintain the physiological pH under acidic conditions (Blankenhorn et al., 1999; De Biase et al., 1999). A unique feature of plant and yeast GAD is the presence

of a calmodulin (CaM)-binding domain in the C-terminal region. In *Saccharomyces cerevisiae*, GAD expression is required for normal oxidative stress tolerance (Coleman et al., 2001). In plants, GAD is thought to be a stress-adapter chaperonin sensing Ca^{2+} signals (Baum et al., 1993; Baum et al., 1996).

3. 2. Two isoforms of vertebrate GAD: GAD65 and GAD67

In vertebrates two molecular forms of GAD have been described, namely GAD65 and GAD67 (referring to GAD with a molecular mass of 65 kDa and 67 kD, respectivly), which are products of two different genes (Fig. 3) (Katarova et al., 1990; Erlander et al., 1991; Bu et al., 1992; Varju et al., 2001). Mouse GAD65 and GAD67 genes are located on chromosome 2. Multiple sequence alignment of GAD65 and GAD67 originated from various species indicate that GAD isoforms show higher homology among species than between isoforms, but the extensive sequence identities of the highly conserved exon-intron structure of the two GAD genes suggest, that both GAD genes were derived from a common ancestral gene by duplication at some point during vertebrate evolution (Erlander et al., 1991; Bu et al., 1992; Lee et al., 1993; Bu and Tobin, 1994; Bosma et al., 1999).

GAD65 and GAD67 are each composed of two major sequence domains called the N-terminal (showing only 23% sequence identity) and C-terminal (showing 73% sequence identity) domains. The N-terminal domain appears to be responsible for the subcellular targeting and formation of GAD65 and GAD67 homo- and heteromers, whereas the C-terminal domain contains the cofactor-binding site and is thought to perform catalytic functions (Sheikh and Martin 1996; Soghomonian and Martin 1998; Kanaani et al., 1999).

In the nervous system, both GAD isoforms are present in most, if not all GABAergic neurons, but display distinct ratios in different brain regions. Based on structural differences, the two forms of GAD differ in cofactor binding, in kinetic properties and subcellular localization. GAD67 associates strongly with the cofactor and makes up most of the constitutively active apo-GAD pool, whereas GAD65 binds PLP loosely and forms most of the inducible apo-GAD (Kaufman et al., 1991; Martin and Rimvall, 1993; Martin, 2000). They also show different subcellular localization: GAD65 is preferentially localized to nerve endings, GAD67 enriched in the cytoplasm (Kannani et al., 1999). GAD67 is thought to preferentially synthesize mostly cytoplasmic GABA for non-synaptic release and metabolic functions, while GAD65 synthesizes GABA for vesicular release that mediates fast-acting synaptic communications (Sheikh and Martin, 1996; Soghomonian and Martin, 1998). The above data suggest that the two GAD enzymes may have distinct roles in neuronal function through establishing functionally different GABA pools.

3. 3. Alternative splicing of GAD67 gene: embryonic GADs

During embryonic development, two alternatively spliced GAD67 transcripts are also expressed that encode the truncated proteins, GAD25 and GAD44 (Fig. 3) (Szabo et al., 1994). The embryonic transcripts include two almost identical alternatively spliced exons inserted into coding sequence of GAD67 mRNA (Bond et al., 1990; Szabo et al., 1994). These exons contain an overlapping stop/start codon, which splits the GAD67 open reading frame into two overlapping ones, one coding for GAD25 and the other for GAD44. These two proteins are colinear with the corresponding regions of GAD67, except for the embryonic exon derived amino acids, found at their C- and N-termini. GAD25 corresponds to the N-terminal regulatory domain of GAD67 and its function has not yet been determined, whereas GAD44 is roughly identical with the C-terminal catalytic domain of GAD67 and synthesizes GABA (Szabo et al., 1994; reviewed in Varju et al., 2001).

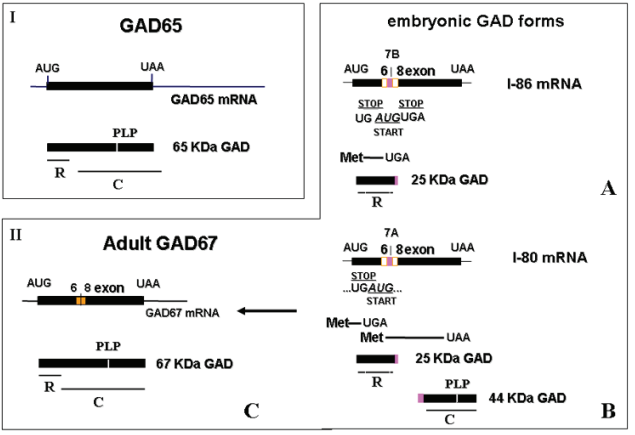

Fig. 3. GAD family. I. GAD65mRNA codes for the 65-kDa GAD protein. GAD65 contains the N-terminal regulatory (R) and the C-terminal catalytic (C) domain needed for co-factor binding (PLP-pyridoxal phosphate). II. Transcripts encoded by the GAD67 gene and their protein products. A. I-86 transcript contains an overlapping STOP/START codon located in exon 7B followed by an additional in frame STOP codon. I-86 mRNA codes for the 25-kDa GAD and contains the regulatory N-terminal domain of GAD67. B. I-80 transcript contains exon 7A wih overlapping STOP/START signal and encodes both GAD25 and GAD44. GAD44 contains the C-terminal catalytic site of GAD67. C. GAD67 mRNA does not contain the embryonic exon 7A/B and codes for the adult full-length GAD67.

The bifunctional embryonic GAD transcript I-80 that contains exon 7A encodes both GAD25 and GAD44. I-86 transcript with additional 6-bp insert at the 3' end of exon 7B contains a further downstream in-frame stop codon that interrupts the translation of GAD44, thus it codes only for the GAD25 protein (Fig. 3) (Szabo et al., 1994; reviewed in Varju et al., 2001).

3. 4. Expression of GAD genes during embryonic development

The two GAD genes are expressed in spatially restricted, largely overlapping domains of the rodent embryonic CNS that further develop into structures with strong GABAergic contribution.

The embryonic GAD mRNAs and their protein forms are expressed throughout the embryonic and postnatal development of the nervous system in a highly specific temporal fashion (Behar et al., 1994; Szabo et al., 1994; Katarova et al., 2000). In the developing mouse telencephalon, embryonic GAD transcripts are transiently expressed in the VZ (ventricular zone) and SVZ (subventricular zone), at sites with active neurogenesis and also at sites containing migratory and postmigratory neuroblasts (Behar et al., 1994; Katarova et al., 2000). The GAD25 form is more abundant in midgestation mouse embryos (E10.5-E12.5), while GAD44 is characteristic for later developmental stages (E12.5-E15.5) (Szabo et al., 1994).

In parallel with neuronal maturation and synaptic formation embryonic forms are gradually replaced by the adult GAD67 as well as GAD65. The adult GAD67 is almost undetectable at E10, but start rising dramatically at birth and reaches adult levels at 4 weeks, which coincides roughly with the end of inhibitory synaptogenesis (Szabo et al., 1994). This implies that embryonic GAD proteins may be involved in the synthesis of morphogenic GABA, on the other hand adult GADs provide GABA for synaptic and metabolic action in mature GABAergic neurons.

Both GAD genes are transiently expressed outside the embryonic central nervous system in structures that are derivatives of the neuronal crest and the placodes indicating that GABA also plays a role in the development of non-neuronal tissues (Katarova et al., 2000; Maddox and Condie, 2001).

3. 5. Upstream regulators of the GAD genes: Dlx2 and Dlx5

The expression of the Distal-less (Dlx) homeobox genes is closely associated with neurons that express GABA in the embryonic brain. Dlx homeobox gene family consists of six known murine members (Stock et al., 1996; Liu et al., 1997; Panganiban and Rubenstein, 2002). Four members have been implicated in neurogenesis: Dlx1, Dlx2, Dlx5, and Dlx6 (Bulfone et al., 1993; Simeone et al., 1994; Anderson et al., 1997a; Liu et al., 1997; Zerucha et al., 2000).

Dlx2 and Dlx5 transcriptional factors are suggested to be upstream regulators of the GAD genes in the developing CNS (Anderson et al., 1997a, 1997b; Stuhmer et al., u 2002a;

2002b; de Melo et al., 2003), where Dlx2 precedes in expression Dlx5 (Eisenstat et al.,1999; Liu et al.,1997).

In addition, Dlx2 and Dlx5 are involved in regulation of developmental processes in a variety of nonneuronal tissues, teeth, vibrissae and palate, in which GAD is also expressed (reviewed in Depew et al., 2005; Levi et al., 2006; Maddox and Condie, 2001; Tamayama et al., 2005; Thomas et al.,1997). The tight correlation of Dlx2 and Dlx5 with GAD expression in these tissues closely mirrors the relationship of Dlx to GABAergic neuronal differentiation, signifying a conservation of function of Dlx genes wherever they are expressed in developing CNS.

4. Other components of GABA signaling

4. 1. GABA receptors as mediators of GABA action

There are two classes of GABA receptors (GABAR) both in the CNS and in non-neuronal tissues: ionotropic $GABA_A$, $GABA_C$ and metabotropic $GABA_B$ (Fig. 4). GABA exerts its effects by different mechenisms and on different time scales depending on whether its action is mediated by ionotropic $GABA_A$, $GABA_C$, or metabotropic $GABA_B$ receptors. The $GABA_A$ and $GABA_C$ receptors are ligand-gated Cl^- channels leading to increased membrane Cl^- conductance that mediate in general rapid synaptic inhibition (Kaila, 1994). $GABA_B$ receptors are coupled to G-proteins and mediate slow onset and prolonged effects of GABA in the central nervous system by indirectly altering membrane ion permeability and neuronal excitability (Bormann, 1988; Kerr and Ong, 1995; Couve et al., 2000).

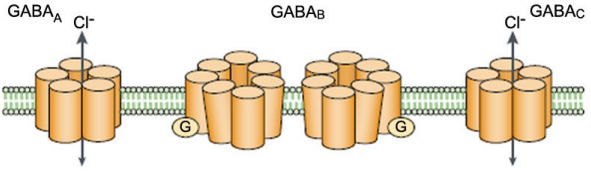

Fig. 4. GABA receptors. $GABA_A$ and $GABA_C$ receptors are closely related pentameric ionotropic receptors. $GABA_A$ receptors are composed of combinations of several subunit types, $GABA_C$ receptors are composed of only single or multiple ρ-subunits. $GABA_B$ receptors are metabotropic receptors that exist as R1 (R1a and R1b isoforms) and R2 subunits, and are associated with G proteins. Native GABAB receptors are dimers composed of one R1 subunit and the R2 subunit. Modified from Ref.: *Owens and Kriegstein, 2002-Nature Neuroscience*

In mammals, among ionotropic GABA receptors, so far six different subunit families have been identified. The majority of fast synaptic inhibition in the brain is mediated by the action of GABA at the $GABA_A$ receptor, a heteropentameric anion-selective channel assembled from various combinations of subunits [α (1-6), β (1-4), γ (1-3), δ, ε, θ, π] (Sieghart et al., 1999). Three additional subunits, ρ (1-3), have been classified as part of the GABAC

receptor (Bormann et al., 1995; Sieghart et al., 1999). Additional diversity of receptor structure is generated by alternative splicing of some of these subunit mRNAs. Based on the presence or absence of a short polypeptide sequence in the second intracellular loop, long and short splice variants, designated L and S, respectively, have been reported for γ2, β2 and β4 subunits (Burt and Kamatchi, 1991). Alternative splicing also results in two different extreme amino-terminal ends for the β3 subunit (Bateson et al., 1991; Lasham et al.,1991).

In the CNS $GABA_A$ subunit expression differs at a regional and cellular level, where subunit composition determines the functional and pharmacological properties and localization (synaptic versus extra-synaptic) of the receptor. The potential combinations of at least 23 subunit forms create a bewildering array of potential subunit combinations; however, a restricted number of subunit combinations assemble together and are properly packaged, processed, and trafficked to the cell surface (Barnes, 2000). In the mammalian central nervous system, the GABAR is belived to be composed of α, β, or β and θ plus one or more of the γ, δ, ε subunits (Sieghart et al., 1999). The α1β2γ2 combination accounts for approximately 43% of $GABA_AR$, although invidiual subunits display distinct regional neuronal and subcellular localization characteristics (McKernan and Whiting, 1996; Nusser et al., 1998; Pirker et al., 2000).

The metabotropic $GABA_BR$ belongs to the G-protein-coupled seven-transmembrane receptor family. The active receptor is a heterodimer of two subunits $GABA_BR1$ and $GABA_BR2$ of which R1 binds GABA and R2 is associated with G proteins (Hill and Bowery, 1981; Jones et al., 1998; Kaupmann et al., 1998; White etal., 1998).

These two receptor subunits are largely co-expressed in the rat brain and combine to form a functional heterodimeric receptor via 'coiled-coil' domains present in the intracellular C-terminal (Bettler et al., 1998; Kuner et al., 1999). The $GABA_BR1$ receptor subunit exists as two, R1a and R1b, isoforms (Kaupmann et al., 1997). Functional native $GABA_B$ receptors seem to be heterodimers that are composed of the R1a or R1b subunit, and the R2 subunit (Kaupmann et al., 1998). Distribution of these two receptor subunit mRNAs in rat tissues showed that both were expressed in the same neurons (Jones et al., 1998; Kaupmann et al., 1998).

In the mammalian central nervous system, $GABA_B$ receptors mediate slow pre- and postsynaptic inhibition. This receptor generates a slow inhibitory postsynaptic potential (IPSP) via coupling to K^+ channel activation (Dutar and Nicoll, 1988; Luscher et al., 1997) or inhibiting neurotransmitter release via, for example, negative modulation of presynaptic Ca^{2+} channels (Scholz and Miller, 1991).

Active GABA receptors are already present in the developing nervous system, however

their subunit compositions differ from that of the adult counterparts. Several $GABA_A$ subunit transcripts have been described in situ as components of functional GABA receptor in rat neuroepithelial cells, as well as in neuroblasts and glioblasts, during spinal cord and cortical neurogenesis (LoTurco et al., 1995; Ma and Barker, 1995; Ma and Barker., 1998; Serafini et al., 1998a, 1998b).

Co-localization of GABA and its receptors were also reported in several adult and developing non-neuronal tissues. Several studies showed that in rat pancreas GABA can increase insulin secretion probably via $GABA_A$ receptor activation (von Blankenfeld et al., 1995; Gu et al., 1993). Immunohistochemical analysis showed co-localization of GABA and its $GABA_A$ and $GABA_B$ receptor subunits in hypertropic-zone chondrocytes of the rat tibial growth plate (Tamayama et al., 2005). The presence of GABA and its receptors in the mouse and human airway epithelium, and in the mouse palate has also been reported (Xiang et al., 2007; Wee and Zimmerman, 1985; Hagiwara et al., 2003).

4. 2. GABA transporters

The vesicular GABA transporter (VGAT) loads GABA or glicine into synaptic or synaptic-like vesicles via catalizing the exchange of one H^+ for one neutral amino acid (Fig. 1), in the embryonic (Oh et al., 2005) and adult brain (McIntire et al., 1997; Wu et al., 2007), pancreatic β-cells (Gammelsaeter et al., 2004) and testis (Geigerseder et al., 2003).

Plasma membrane GABA transporters are high-affinity, sodium (Na^+) and chloride (Cl^-)-dependent transporters, and GABA is co-transported with Na^+ and Cl^- (Borden, 1996; Gadea and Lopez-Colome 2001; Conti et al., 2004). Molecular cloning has revealed four GATs, GAT1-4. In the adult CNS, GATs remove GABA from the synaptic cleft into presynaptic neurons or surrounding glia (Schousboe, 1983) (Fig. 1). GATs were found in many peripheral tissues outside the CNS. GAT-1 was found existing in the testis and sperm of rat and mouse (Ma et al., 2000; Hu et al., 2000), GAT-2, GAT-3 and GAT-4 in liver and kidney of mouse and human (Rasola et al., 1995; Jursky and Nelson, 1999; Gadea and Lopez-Colome, 2001).

Neuronal membrane GABA transporters could also be involved in non-synaptic GABA release by reverse operation in the embryonic nervous system and in the retina (Attwell et al., 1993; Levi and Raiteri, 1993; do Nascimento et al., 1998). In cultured chick retina cells pharmacological experiments revealed that the uptake and release of GABA are differentially inhibited by GAT-1 and GAT2/3 blockers indicating that sodium dependent GABA release from cultured chick retina cells was mediated by a GAT-1 like transporter (Do Nascimento et al., 1998).

5. GABA and GAD in non-neuronal cell types

GABA and GAD, in addition to the CNS, are present in a number of non-neuronal tissues. GADs and most probably GABA are also expressed transiently in non-neural tissues during development and may be required for the normal development (Hagiwara et al., 2003; Katarova et. al., 2000; Maddox and Condie, 2001).

GABA and GAD have been found in chromaffin cells of the adrenal gland, platelets and erythrocytes of blood, endocrine cells of the gastrointestinal tract and the pancreas, tubular epithelium of the kidney, hepatocytes, pinealocytes, germinal cells and spermatocytes of the testis, oocytes of ovary, epithelial cells of the oviduct, chondrocytes of the growth plate, the apical membranes of airway epithelial cells and keratinocytes (Kataoka et al., 1984; Gilon et al., 1988; Erdö and Wolff, 1990; Farquharson and Loveridge, 1990; Gilon et al., 1991, 1992; Tillakaratne et al., 1992, 1995; Tamayama et al., 2005; Xiang et al., 2007). GABA in non-neural tissues may play a multitude of functions such as regulation of motility and agglutination of spermatozoa, stimulation of testosterone production, regulation of uterotubal and gut motility, and control of hormone release in endocrine cells in the adrenal medulla, pancreas and gastrointestinal tract (Kataoka et al., 1984; Erdö and Bowery, 1986; Racagni and Donoso, 1986; Gilon et al., 1988, 1992; Tillakaratne et al., 1995).

GABA and GADs were shown to be present in developing non-neural tissues such as the tail bud mesenchyme, the pharyngeal pouches and arches, the ectodermal placods of the developing vibrissae, the apical ectodermal ridge, mesenchyme and ectoderm of the limb buds, olfactory epithelium, heart and endothelial cells of blood vessels (Katarova et. al., 2000; Maddox and Condie, 2001). The cleft palate phenotype, of the GAD67 knock-out mutants and mice with a deletion or targeted mutation in the β3 subunit of the $GABA_A$ receptor, showed the involvement of GABA-mediated signals in the normal development and differentiation of palate, the structure derived from the oral epithelium and neural crest ecto-mesenchyme (Maddox and Condie, 2001; Hagiwara et al., 2003).

The dynamic expression of GABA and GAD in such multifarious tissues suggests a wider role for GABA signaling in development than was previously appreciated. The metabolic pathway involved in GABA synthesis is common to most, if not all, procaryotic and eucaryotic cells and is not restricted to neurons. The metabolic pathways and the components of the GABA signaling system are present in a broad range of cells, raising the possibility that activation of signaling cascades through this amino acid could be a phylogenetically conserved ubiquitous mechanism.

The exact function of the GABAergic system outside the central nervous system is still unknown. The details of proposed mechanisms remain to be elucidated. The difficulties

originate mainly from the widespread distribution of GABA and the variety of functions it may exert. However, more insights can be provided by transgenic animal models, in which individual components of the GABA signaling pathway could be altered even in different cell types and in a temporarily regulated fashion.

6. GABA signaling: focus on development and pathological conditions

In the adult vertebrate central nervous system GABA functions primarily as an inhibitory neurotransmitter, while it is considered to act as a trophic factor during development, regulating key developmental steps in the embryonic nervous system, such as proliferation, migration, differentiation, synapse maturation and cell death (LoTurco et al., 1995; Barker et al. 1998; Katarova et al. 2000; Owens and Kriegstein, 2002; Ben-Ari, 2002). In the developing embryo, GABA has been found to play an important role in the morphogenesis and maturation of many tissues outside the nervous system. Studies have also revealed that GABA participates in regulating cell division and affects the differentiation and maturation of cell in humans as well as in rodents (Gilon et al., 1987; Maddox and Condie, 2001; Tamayama et al., 2005; Watanabe et al., 2002). Moreover, several reports recently showed that expression of GABA and GAD significantly increased in neoplastic tissues, such as colorectal carcinoma, breast cancer, and gastric cancer, as compared with normal tissues (Kleinrok et al., 1998; Matuszek et al., 2001; Watanabe et al., 2002; Watanabe et al., 2006; Maemura et al., 2003).

6. 1. GABA depolarizes immature neurons

With respect to the involvement of GABA in the development of neuronal structures, an increasing amount of evidence indicated that GABA acts in an excitatory manner via ionotropic GABA receptors during development to aid migration, proliferation, differentiation of neurons, the establishment and refinement of synaptic connections (Cherubini et al., 1988; Cherubini et al., 1991; Strata and Cherubini 1994; Ben-Ari et al., 1997; Varju et al., 2001).

During brain development, $GABA_A$ receptors are operative before synapse formation, suggesting that their action is not restricted to synaptic transmission (Meier et al., 1983; Lipton and Kater, 1989). Activation of $GABA_A$ receptors leads to opening of channels that are permeable to Cl^- and HCO_3^-. Under physiological conditions, the corresponding currents have a reversal potential (E_{GABA}) close to the neuronal resting potential (Fig. 5). In recent years, it has become increasingly clear that the resulting effects on the postsynaptic cell can be either inhibitory or excitatory. In particular it has been realized that E_{GABA} is developmentally regulated such that activation of $GABA_A$ receptors is mostly excitatory in

neonatal brain preparations, but becomes inhibitory later in development (Fig. 5) (Bormann et al., 1987; Delpire, 2000; Ben-Ari 2002; Owens and Kriegstein 2002). Excitatory versus inhibitory effects of GABA depends on intracellular chloride concetration, which is high in immature neurons and becomes low in mature neurons, due to the increasing expression of the kalium-chloride co-transporter (KCC2), the chloride extruder. The excitatory action of GABA early in life is presumbly important for neuronal development and network formation.

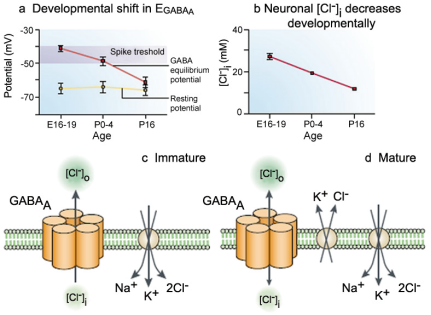

Fig. 5. Developmental shift in GABA actions. **a.** The GABA receptor equilibrium potential (E_{GABA}) decreases during development. **b.** The developmental shift in E_{GABA} is due to a developmental decrease in the $[Cl^-]_i$. **c.-d.** A developmental shift in GABA actions occurs as a result of changing intracellular chloride concentration. Modified from Ref.: *Owens and Kriegstein, 2002-Nature Neuroscience*

The abundance of GABA and its receptors in the early developing rodent CNS both *in vivo* and *in vitro* has led to speculations on the role of this transmitter in immature neural cell proliferation, migration, differentiation and survival (LoTurco et al., 1995; Barker et al., 1998; Owens and Kriegstein 2002, Ben-Ari, 2002). It is now well established that GABA exerts a variety of trophic influence through the stimulation of the $GABA_A$ receptor (reviewed by Barker et al., 1998; Lauder etal., 1998). During a limited period of early neuronal development, when GABA is depolarizing, elevates $[Ca^{2+}]_i$, which mediates the trophic action of GABA in neuronal maturation (Cherubini et al., 1991; LoTurco et al., 1995; Serafini et al., 1998a, 1998b; Owens et al., 1996, 1999). Ca^{2+} exerts a wide variety of effects on the developing brain, where it can modulate proliferation of neural progenitors, migration of neuronal precursors (Behar et al., 2000), the rate and direction of neuritic growth (Mattson and Kater, 1987) and influence gene expression (Vaccarino et al., 1992; Bading et al., 1993).

The Ca^{2+} influx in immature neurons requires the existence of a driving force for a depolarizing Cl^- current. Inward Cl^- transport by Na^+-K^+-$2Cl^-$ cotransport (Plotkin et al., 1997; Sung et al., 2000; Jang et al., 2001) and/or HCO_3^- Cl^- - exchange (Kobayashi et al., 1994; Rohrbough and Spitzer, 1996) have been suggested as the mechanisms underlying the depolarizing GABA response of immature neurons (Fig. 6). NKCC1 (sodium potassium chloride co-transporter), which is driven by sodium and potassium gradients, accumulates intracellular chloride, and is responsible for the high intracellular chloride concentration $[Cl^-]_i$ in dorsal root ganglia and in neocortical neurons (Fig. 6) (Fukuda et al., 1998; Ben-Ari, 2002).

The Ca^{2+} influx in response to GABA disappears with maturation of most neurons (Leinekugel et al., 1995; LoTurco et al., 1995; Chen et al., 1996; Eilers et al., 2001). The change is accompanied by a progressive $[Cl^-]_i$ depletion (Obrietan and van den Pol, 1995; Li et al., 1998). Developmental upregulation of KCC2 results in hyperpolarizing inhibition of hippocampal neurons (Fig. 6) (Rivera et al., 1999). Some mature neurons may, however, also maintain depolarizing responses to GABA throughout their life. An example is provided by dorsal root ganglion cells in which the Cl^- gradient for depolarizing currents is sustained by Na^+-K^+-$2Cl^-$ cotransport (Sung et al., 2000).

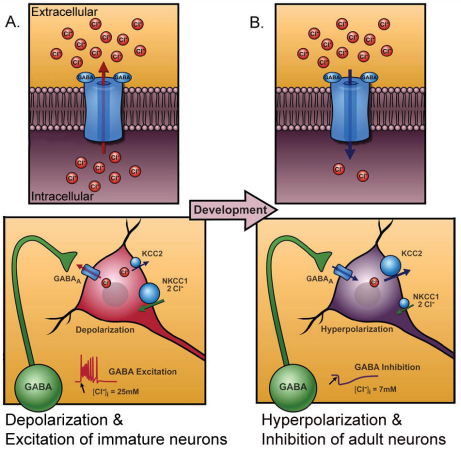

Fig. 6. Early expression of NKCC1 and late expression of KCC2 determines developmental changes in $[Cl^-]_i$. During development, the intracellular chloride concentration decreases due to the changes in the expression of the two major chloride cotransporters, KCC2 and NKCC1. **A.** In immature neurons, an inwardly directed Na^+-K^+-Cl^- cotransporter (NKCC1) acts to maintain relatively high intracellular chloride concentrations. **B.** In mature neurons, intracellular chloride concentration is decreased by the expression of an outwardly directed K^+-Cl^- co-transporter (KCC2). Modified from Ref.: *Ben-Ari, 2007-Physiol. Rev.*

6. 2. GABA as a modulator of proliferation

6. 2. 1. GABA mediates proliferation of neuronal cells

In precursor cells in the rat neocortical proliferative zone, activation of $GABA_A$ receptors has been shown to influence DNA synthesis (LoTurco et al., 1995; Owens et al., 1999; Haydar et al., 2000). Studying tritiated-thymidine [^{3}H-thymidine] or 5-bromodeoxyuridine (BrdU) incorporation in cells derived from the E16-E19 cortex has revealed that micromolar concentration of GABA inhibited DNA synthesis in proliferating cells (LoTurco et al., 1995). The effect was blocked by the $GABA_A$ receptor antagonist bicuculline methiodide and could be mimicked by activating voltage-dependent ion channels by adding depolarizing concentration of KCl. Recent studies point to a more complicated situation. In embryonic mouse cortical slices $GABA_A$ receptor activation led to an increase in the number of BrdU-labelled cells in the VZ, but to a decrease in the SVZ (Haydar et al., 2000). In cerebellar granule cells potassium-induced depolarization could either increase or

decrease cell proliferation depending on the experimental conditions (Borodinsky and Fiszman, 1998; Cui and Bulleit, 1998).

There is no plausible explanation at present for GABAs divergent effects on the cell cycle and the components of the downstream cellular machinery remain to determine. The depolarizing effect of GABA on cell proliferation is most probably due to an elevated intracellular Cl^- concentration, which is particularly high in dividing precursors and decreases with the advance of neuronal differentiation and synaptic formation (Cherubini et al., 1991; LoTurco et al., 1995; Rivera et al 1999; Owens et al., 1996, 1999).

6. 2. 2. GABA regulates proliferation of peripheral nonneuronal cells

There has been little research on the participation of the GABAergic system in the proliferation of nonneuronal cells. Recent results indicate an important role for GABA in protecting and replenishing pancreatic islet cells. In addition to influencing cell survival, GABA also has a role in stimulation of pancreatic islet cell proliferation (Ligon et al., 2007).

Studies with the ATDC5 mouse chondrogenic cell line showed that GABA contributes to cell proliferation via activation of $GABA_A$ and $GABA_B$ receptors (Tamayama et al., 2005).

In the testis, the GABAergic system is involved in the regulation of spermiogenesis (Kanabara et al., 2005). In addition, it has been shown that proliferation of Leydig cells is stimulated by GABA via activation of $GABA_A$ receptors (Geigerseder et al., 2004).

On the other hand, in rat jejunum GABA has an inhibitory effect on epithelial cell proliferation (Wang et al., 2004).

Furthermore, $GABA_A$ mediated growth inhibition of the liver following partial hepatectomy and during recovery from various forms of hepatic injury are also reported (Minuk et al., 1995; Kaita et al., 1998; Watanabe et al., 2006).

The above examples demonstrate that, opposing effects of GABA on cell growth occurs in peripheral nonneuronal cells as well as in neuronal cells.

6. 2. 3. GABA influences cancer cell proliferation

Since GABA has an effect on proliferation of various normal cell types, a role in cancer cell proliferation can also be considered. Increased GAD activity and GABA content have been reported in colon cancer (Kleinrok et al., 1998; Wang et al., 2000; Maemura et al., 2003), breast cancer (Mazurkiewicz et al., 1999; Opolski et al., 2000), prostate cancer (Azuma et al., 2003; Hu et al., 2001), gastric cancer (Matuszek et al., 2001), and glioma (Bianchi et al., 2004). Furthermore, GABA receptor mRNAs and proteins are increased in neuroblastoma (Roberts et al., 2004), liver cancer (Biju et al., 2002), and breast cancer (Jiang et al., 2002).

Exprerssion of GAD65, GABA and $GABA_A$ receptor subunits were observed in gastric cancer cell line KATO III cells. KATO III cells treated with $GABA_A$ receptor agonists and antagonists, including GABA, showed that GABA increases proliferation of these cells via $GABA_A$ receptors (Shiraishi et al., 2007).

Takehar and colleuges reported that GABA stimulates pancreatic cancer growth through overexpressing $GABA_A$ receptor π subunit (GABRP). The addition of GABA into the cell culture medium promoted the proliferation, increased intracellular Ca^{2+} levels in GABRP-expressing pancreatic ductal adenocarcinoma (PDAC) cells, and $GABA_A$ receptor antagonists inhibited this growth-promoting effect by GABA. In addition, clinical PDAC tissues contained a higher level of GABA than normal pancreas tissues due to the upregulation of GAD67 expression (Takehara et al., 2007).

A recent study has shown that the $GABA_A$ receptor agonist, muscimol, dose dependently inhibited epidermal growth factor (EGF) induced DNA synthesis and enhanced the transforming growth factor β1 (TGF β1) mediated DNA synthesis suppression in primary hepatocyte cultures, suggesting that $GABA_A$ receptor act as an inhibitory signal for hepatic cell proliferation (Biju et al., 2001).

These findings imply that GABA in concert with other signaling components could play important roles in cancer development and progression, and that this pathway can be a promising molecular target for the development of new therapeutic strategies.

6. 3. Modulatory role of GABA in cellular motility

6. 3. 1. GABA regulates neuronal migration

In vivo, GABA is detected near the target destinations of migratory neurones (Lauder et al., 1986; Del Rio et al., 2000; Katarova et al., 2000) and also in migratory neurones themselves (Taylor et al., 1990; Taylor and Gordon-Weeks 1991; Bless et al., 2000; Del Rio et al., 2000) during neuronal development. Further analysis has revealed that $GABA_C$-like receptors regulate the migration from the VZ to the IZ (intermediate zone) while activation of $GABA_B$ receptors contributes to the migration of cells from the IZ towards the CP (cortical plate) (Behar et al., 2000). $GABA_A$ receptor activation has been proposed to provide a „stop signal" for migrating neurones as $GABA_A$ receptor agonist bicucullin causes thickening of the CP due to an increase in the number of migrated neurones (Behar et al., 2000). Blocking of GABA receptors resulted only in a delay of cell movements, but not a complet arrest of migration, indicating that GABA receptor activation seems not to initiate, but only to modulate, the rate of cell migration in the developing cortex (Behar et al., 2000). Furthermore, other factors have also been reported to play a role in the arrest of cell migration

at the cotical plate and establishment of the cortical layering (Ogawa et al., 1995; Super et al., 2000; Dulabon et al., 2000).

GABA has been reported to inhibit the migration of early LHRH (luteinizing hormone-releasing hormone) progenitors, a subpopulation of which express GABA during migration. Recent data suggests that this effect is mediated by $GABA_A$ receptors, although the underlying molecular events are largely unknown (Fueshko et al., 1998; Bless et al., 2000; Lee et al., 2008)

Studies have shown that both R1 and R2 subunits of the $GABA_B$ receptor are present in the embryonic cortex, and that $GABA_B$ receptor activation can influence the movement or migration of immature cortical neurons, indicating that $GABA_B$ receptors appear to have developmental functions (Behar et al., 1996, 2000, 2001; Maric et al., 2001).

The GABA-mediated migratory signals have been suggested to act through Ca^{2+} transients that alter cell movements through changing the dynamics of cytoskeletal remodelling (Gomez et al., 1995; Gomez and Spitzer 1999).

6. 3. 2. GABA as a regulator of cancer metastasis

GABA has been reported to reduce the migratory activity of SW480 colon cacinoma cells by activating $GABA_B$ receptors and decreasing cAMP concentaration (Joseph et al., 2002). In human breast cancer cells, migration was stimulated by the $GABA_A$ receptor agonist propofol (Garib et al., 2002).

Increased GABA and GAD expression was found in prostate cancer patients with metastasis, but not in those without metastasis or in patients with BPH (benign prostatic hypertropy). It has been shown that GABA promotes cancer cell invasion via $GABA_B$ receptor pathway, and an increase in MMP (matrix metalloproteinase) activity is an underlying mechanism of action (Azuma et al., 2003).

6. 4. GABA as a regulator of neuronal differentiation

In addition to proliferation and migration, aspects of neuronal differentiation might be regulated by early GABA-mediated signaling. In cultured embryonic hippocampal and neocortical neurons, $GABA_A$ receptor activation has been shown to promote neurite outgrowth and maturation of GABA interneurons (Barbin et al., 1993; Marty et al., 1996; Maric et al., 2001). In this particular case, GABA might exert its trophic effects by stimulating an increase in brain-derived neurotrophic factor (BDNF) expression and release from target neuron (Berninger et al., 1995; Marty et al., 1996).

Recent observations suggest that the maturation of hyperpolarizing GABAergic activity

could be required for synaptogenesis and for establishing circuits whose development depends on mechanisms such as spike-timing-dependent plasticity. Because GABA depolarizes immature neurons throughout the period of their morphological development and synaptogenesis, membrane depolarization in response to GABA probably represents a fundamental signaling mechanism regulating neuronal development and circuit assembly (Akerman and Cline, 2007; Wang and Kriegstein, 2008).

7. The vertebrate eye lens

Since the dissertation is based on studies of GABA signaling in developing mouse lens, this chapters briefly summarize the major events of lens development and the underlying mechanisms.

7. 1. Overview of mouse lens development

The lens is a highly specialized, transparent, biconvex (outward curve on both sides) structure inside the eye that, along with the cornea, helps to refract light to be focused on the retina. The lens, by changing shape, functions to change the focal distance of the eye so that it can focus on objects at various distances, thus allowing a sharp real image of the object of interest to be formed on the retina.

The lens, because of its simplicity, predictable pattern of development and differentiation, has attracted the attention of many developmental biologists.

Development of the vertebrate eye becomes first evident, when the lateral walls of the diencephalon begin to bulge out as optic placodes, they enlarge to form the optic vesicles, which teminate very close to the overlying surface ectoderm (Fig.A). In E9.5 mouse embryos the outer surface of the optic vesicles retract inward to form the optic cups, while the surface ectoderm in the localized regions overlying the outer border of the optic cups is induced to form the lens placod (Fig.B). There is experimental evidence that optic vesicle provides a signal for the induction of the lens placod (Kaufman, 1992; Piatigorsky, 1981; Lang, 2004). After the lens vesicle pinches off from the surface head ectoderm (Fig.C, D), its cavity is obliterated by the elongation of the epithelial cells at the posterior lens vesicle (Fig.E). This first population of lens fiber cells is called primary lens fiber cells. The cells at the anterior pole of the lens vesicle remain as a single layer of cuboidal epithelial cells. The lens epithelium has the potential to divide, particularly in the germinative zone close to the equator that forms a boundary between the epithelium and the fibers. In this so called, transitional zone the cells are pushed into the posterior compartment where they cease mitosis, elongate, and constitute secondary lens fibers surrounding the primary lens fibers. Succeeding

generations of secondary fibres are added in concentric shells at the periphery of the primary fibre cell mass (Fig. F, G).

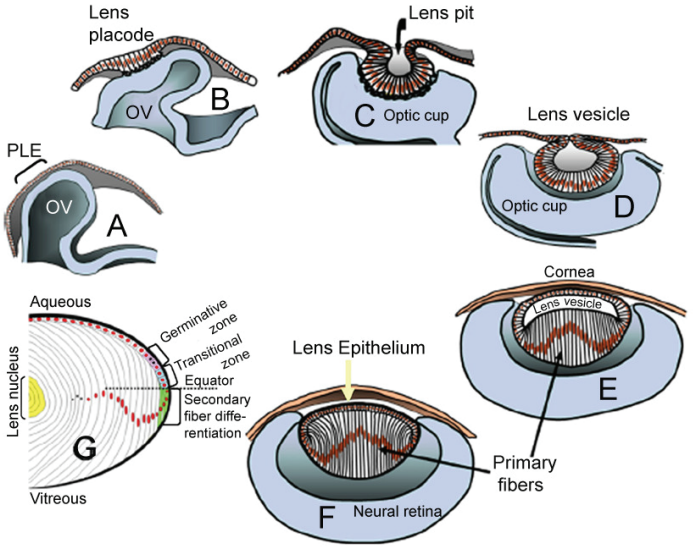

Fig. 7. Schematic view of a developing mouse eye. A. Morphological development of the lens begins as the optic vesicle (OV) approaches the presumptive lens ectoderm (PLE). **B.** Cells within the PLE elongate forming the lens placode (E9.5). **C.** The lens placode invaginates forming the lens pit and the OV invaginates forming the optic cup (E10.5). **D.** The lens pit deepens and the connection of the lens pit and overlying surface ectoderm is lost forming the lens vesicle. **E.-F.** Once the lens vesicle has formed (E11.5) the primary lens fibers elongate from the posterior epithelium of the lens vesicle and fill its entire lumen. **G.** The mature lens consists of an anterior epithelial layer composed of non-proliferating central lens epithelial cells and a narrow band of proliferating cells known as the germinative zone (pink cells). Just posterior to the lens equator transitional zone (blue cells) epithelial cells begin to elongate forming secondary fiber cells (green cells). The secondary fiber cells start to elongate at the lens bow region (equator) at E14.5. The lens nucleus (yellow) is composed of fiber cells that were present in the embryonic lens. Modified from Ref.: *Robinson, 2006-Cell and DevelopmentalBiology*

The ocular lens grows throughout life in both diameter and cell number. At the equator, the epithelial cells begin to elongate, lose their intracellular organelles (Bassnett et al., 2002), synthesize the crystallins (Piatigorsky, 1989) and thus differentiate into fiber cells. Both, the primary and secondary fiber cells lose their organells including mitochondria and cell nuclei during the final differentiation process. For the primary fibers, it takes place in mice at E17/E18 and is finalized 2 weeks after birth, when the mice open their eyelids (Vrensen et al., 1991). The secondary fiber cells, which encircle the primary fiber cells, lose their organelles, when they move from the outer to the inner cortex (Kuwabara, 1974). In the process of differentition, fiber cells elongate and migrate bidirectionally until their tips reach a point where they encounter fiber cells from the opposite hemisphere of the lens. When elongation is completed, fiber cell tips detach from the epithelium or capsule and overlap with tips of

opposing fiber cells (Kuszak et al., 1984, 2004). The overlap of tips from opposing cells forms a seam referred to as a suture line (Kuszak et al., 2004). Shortly after reaching the sutures, fiber cells detach from the lens capsule and are covered by the next cohort of differentiating cells (Kuszak et al., 1984). Differentiation of lens epithelium to fibers, both during development and in the mature lens, is characterized by appearance and subsequent massive accumulation of the cytoplasmic crystallins, α, β, γ in mammalians, and of an integral membrane protein known as the major intrinsic protein (MIP) (Alcala et al., 1975; Bloemendal et al., 1989, Bhat, 2003; Bloemendal et al., 2004).

The lens is an avascular tissue, separated from the aqueous and vitrous humors by its own extracellular matrix, the lens capsule. The lens capsule is a specialized thickened basement membrane that completely surrounds the lens and provides anchoring sites from zonules, the filamentous bodies that suspend the lens. An elastic lens capsule may be necessary in order to accomodate rapid lens growth in early development, whereas later in development a stronger, more cross-linked capsule may be necessary in order to tolerate the stress caused by postnatal accomodation and disaccomodation of the lens (Kelley et al., 2002).

7. 2. Multiple factors controlling lens development

A multi-factorial program of events establishes and maintains the basic lens growth pattern. Distinct cellular processes during lens development and growth are dependent on the tight spatial and temporal regulation of cell proliferation and differentiation controlled by distinct transcription and growth factor-induced signaling pathways. A number of transcription factors have recently been identified as regulators of eye and lens development, including homeobox genes Pax6, Six3, Prox-1 and retinoic acid receptors (Cvekl and Piatigorsky, 1996; Fini et. al., 1997, reviewed in Ogino and Yasuda, 2000). The fibroblast growth factor (FGF) appears to be a key initiator of fiber differentiation (Chamberlain and McAvoy, 1987), but a number of studies have also shown that other growth factors, such as TGFa, PDGF-A (platelet-derived growth factor), IGF-1 (insulin-like growth factor), EGF and HGF (hepatocyte growth factor) are also involved in lens fiber cell proliferation (Hyatt and Beebe, 1993; Wunderlich et al., 1994; Kok et al., 2002; Choi et al., 2004; reviewed in Lovicu and McAvoy, 2005). Although eye development is one of the most extensively studied processes in the embryo, the identity and specific role(s) of many regulatory factors controlling lens differentiation is still obscure.

7. 3. Lens as a model system

The vertebrate lens provides an ideal model for studying complex signaling pathways. All stages of lens differentiation, from the proliferation of lens epithelial cells to the differentiation of mature, organelle-deficient fiber cells, are represented in the lens of any individual. Analogous to the rings of a tree, the components of the lens, from the newest cells born moments before tissue collection to the oldest cells originating during embryogenesis, offer a key to its life history. One of the features of the lens that makes it attractive as a model system is its regional compartmentalization. Proliferation occurs in the anterior lens epithelium, migration in the equatorial zone, and differentiation at the posterior pole of the lens. The lens is an exquisite developmental model to study the involvement of the GABAergic system in these developmental processes.

III. Specific aims

The experimental work, part of which is included in this dissertation initiated in 2000, in the laboratory of Dr. Gábor Szabó at the Department of Gene Technology and Developmental Neurobiology, Laboratory of Molecular Biology and Genetics, Institute of Experimental Medicine, Budapest, Hungary.

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 $GABA_A$ and $GABA_B$ receptor subunits, vesicular and membrane GABA transporters.

(3) Establishment and characterization of primary mouse lens epithelial cell culture to study GABA signaling *in vitro*.

(4) Testing the functionality of GABA signaling in the lens by calcium-imaging using confocal laser microscopy.

- To study the effect of external GABA on intracellular calcium levels in intact lens
- To study in primary mouse LEC the effect of $GABA_A$ and $GABA_B$ receptor activation on intracellular calcium levels

(5) Generation and characterization of transgenic mice that overexpress GAD67 under the control of lens specific αA-crystalline promoter.

- Verifying transgene expression and determining GAD67 and GABA levels in the lens of CrysGAD67 transgenic mice.
- Characterization of the phenotypic changes in transgenic lens overexpressing GAD67.

(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.

IV. Materials and 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 and GAD65 and GAD67 single and double knock-out (KO) mice, which were housed in the SPF animal facility of the Institute of Experimental Medicine.

All experiments with animals were conducted in compliance with institutional, NIH (NIH Publication #85-23, 1985) and EC (86/609/EEC/2) guidelines and approved by in-house and national committees.

To obtain embryonic tissues for histology, female mice were individually mated overnight with males. The presence of a vaginal plug was considered as embryonic day 0.5. Newborn pups were marked as P0 (postnatal day 0).

2. Genetically modified mouse lines used in this study

GAD65-GFP transgenic mice were made earlier in our laboratory and will be described elsewhere (Erdelyi et al., in preparation). Line GAD65_3.gfp5.5 #30 used in this study contains 5.5 kb of the GAD65 promoter fused to eGFP in the third exon such that the GAD-GFP protein contains the first 87AA of the NH2-terminus of GAD65. GFP marker is specifically expressed in GABAergic neurons in most of the brain regions and in several non-neuronal tissues including the lens. This line was maintained in heterozygous form on FVB/N genetic background. Transgenic newborns and embryos used in this study were identified under UV light by direct visualizing GFP fluorescence through the scull with special goggles equipped with appropriate filter.

GAD67-GFP knock-in/GAD65 knock-out mouse line was obtained from Yuchio Yanagawa (Gunma University Graduate School of Medicine, Maebashi, Japan), and single GAD65KO and GAD67KI mice were generated from the double knock-out line through genetic segregation. GAD65+/- GAD67+/- double heterozygous knock-out mice were used as the breeders to produce GAD65-/-/GAD67-/- double knock-out embryos. GAD65-/- GAD67-/- mice were obtained at low frequency and did not survive after birth because of cleft palate, characteristic of GAD67-/- mice (Asada et al., 1997). GAD65KO and GAD67KI genotypes were determined using polymerase chain reaction as reported previously (Asada et al., 1996, 1997).

CrysGAD67 transgenic mice were generated within the scope of the study outlined here.

3. Generation of transgenic mice

Cloning was performed according to established procedures (Sambrook et al., 1989).

The alpha crystallin promoter vector used in this study (CPV2-a modified version described in Robinson et al., 1995) carries the murine αA-crystalline promoter (Overbeek et al., 1985), and a polyadenylation signal sequences of the SV40 virus early region (Gorman et al., 1982) in Bluescript KS- general cloning vector (Stratagene).

GAD67 cDNA was ligated to the *HindIII-ClaI* digested pβactin-hCGFP vector (Fig. 8A-a gift from A. Matus), which resulted in an inframe fusion of GAD67 and green fluorescent protein (GFP). To make the Crys-GAD67-GFP transgenic construct, GAD67-hCGFP fusion gene was released from the vector as a *HindIII-XbaI*-blant ended DNA fragment, then inserted into *HindIII-blunt-ended EcoRI* sites of the αA-crystallin promoter vector CPV-2 (Reneker et al., 1995) (Fig. 8B) between the promoter and the splicing/polyadenylation signals from the early region of SV40 virus (Fig. 8C).

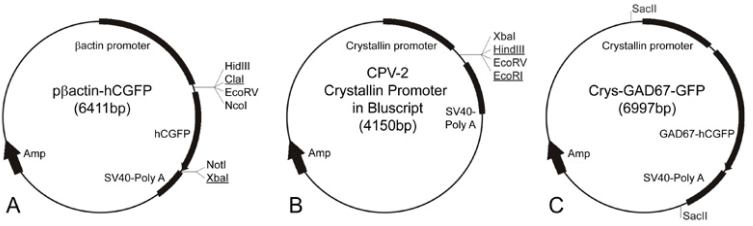

Fig. 8. Schematic presentation of the vectors used in construction process of plasmid Crys-GAD67-GFP. The structure of pβactin-hCGFP (A), αA-crystallin promoter vector 2 (CPV-2) (B) and Crys-GAD67-GFP plasmid (C).

αACrys-GAD67-GFP plasmid construct was linearized (with *SacII*) and gel purified by electroelution, phenol/chloroform extracted and resuspended in 10mM Tris-HCl (pH 7.5), 0.25 mM ethylendiaminetetraacetic acid (EDTA) (Fig. 9). 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 P^{32}-labeled SV40 fragment as a transgene specific probe. Heterozygous lines were established by crossing the founders to FVB/N mice and homozygous lines were generated by mating heterozygous transgenic males and females. CrysGAD67 homozygous mice were selected by Dot blot analysis, backcrossed to wild type mice to prove homozygous status for the transgene.

Progenies were genotyped by transgene specific PCR using tail DNA. Two sets of primers were used in a single reaction: transgene specific SV40 forward primer 5'-AACCACAACTAGAATGCAGTG-3', reverse primer 5'-CTTGCTTTAAAAAACCTCCC-3'

(Fig. 9); control Dlx2 forward primer 5'-GGACAGCGTCTGAGACTTGA-3', reverse primer 5'-TCAGGTCGGAATTGAGGC-3'; the PCR products were 166 bp and 237 bp, respectively.

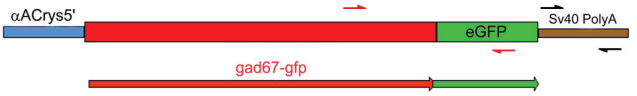

Fig.. 9. αACrys-GAD67-GFP transgenic construct. Schematic drawing of the αACrys-GAD67-GFP microinjection construct. The construct contains: 411bp αACrystallin promoter fragment including 43bp 5'-untranslated region, GAD67 cDNA fused in frame with eGFP coding region and SV40 poly(A) addition signal with an intron. Primers SV40F and SV40R (black arrows), which flank the intron were used to screen genomic tail DNA via PCR. The SV40 sequence was used to generate a probe for Dot blot and a riboprobe for in situ hybridization. Transgene expression was analyzed by using a forward primer toward the 3' end of the mouse GAD67 cDNA and a reverse GFP primer (red arrows).

4. RNA preparation

Lenses were dissected from embryos or deeply anaesthetized mice of various ages in ice-cold DEPC (diethyl pyrocarbonate, Sigma-Aldrich Co.)-treated phosphate-buffered saline (PBS) pH 7.4. The cilliary epithelium, vitreous and retina remnants were removed with fine forceps under a dissecting microscope. Lenses were collected in RNA-later (Sigma-Aldrich Co.) and stored in the same solution at –20ºC. Embryonic and adult mouse brains used as controls were collected in liquid nitrogen and stored at –80ºC.

Total RNA was isolated from tissue using TRI-Reagent (Sigma-Aldrich Co.) following the manufacturer's protocol. The concentration and purity of the RNA preparations were determined by measuring optical density at 260 and 280 nm, the ratio of OD 260/280 being >1.8 for all preparations.

Transgenic total RNA samples were treated with RNase-free DNase I (Promega) to eliminate possible DNA contamination following the recommended procedure. In brief, 4.5 µg of RNA was resuspended in 1µl 10x Reaction Buffer and 4.5U of DNase I into final volume of 10µl. Reaction mixture was incubated at 37ºC for 20 min. The reaction was stopped by the addition of 2mM EDTA, pH 8.0, followed by inactivation at 65ºC for 10 min.

5. Semi-quantitative RT-PCR amplification

Three micrograms of RNA were reverse-transcribed using RevertAid^{TM} H Minus First Strand cDNA Synthesis Kit with random hexamer primers (Fermentas UAB) according to the manufacturer's instructions. PCR amplification was performed on one tenth of the first strand cDNA.

Conditions for PCR reactions were as follows: initial denaturation for 5 min at 95°C, followed by 30 cycles of amplification as follows: 60 sec at 94°C; 60 sec at 58- 60°C; 60-90 sec at 72°C followed by a final extension for 10 min at 72°C. The same amount of total RNA

Table 1. Primers and amplification parameters used in the semi-quantitative RT-PCR analyses

mRNA	Position	Primer Sequence	Product Size (bp)	Annealing temp.(°C)	Extention time (sec)
Actin	609 1107	F 5'-AGCTGAGAGGGAAATCGTGC-3' R 5'-GATGGAGGGGCCGGACTCAT-3'	499	55-62	60-90
Dlx2	138 488	F 5'-CAACAGCAGCCTGCACAA-3' R 5'-GAGTAGATGGTGCGTGGTTTC-3'	351	60	60
Dlx5	186 535	F 5'-CTCTGCTTCTTATGGCAAAGC-3' R 5'-TTTGCGTCAGTCCTAGAGAGG-3'	350	58	60
EGAD	438 681	F 5'-GCTGGAAGGCATGGAAGGCTTTA-3' R 5'-TGAGCCCCATCACCGTAGCA-3'	244	58	60
GAD67	618 1722	F 5'-GACTGCCAATACCAATATGTTCAC -3' R 5'-GGCGGCTGGGTTAGAGATG -3'	1105	60	90
GAD67 *	1842 2195	F 5'-TCATCGGCCATGCTTGTGC-3' R 5'-CCATCCGCCCTGTAGTTGC-3'	353	56	60
GAD65	13 450	F 5'-GGCTCCGGCTTTTGGTCCTTC-3' R 5'-TGCCAATTCCCAATTATACTCTTGA-3'	438	58	80
$GABA_A\alpha1$	51 634	F:5'-TCTGAGCACACTGTCGGGAAG-3' R:5'-ACCCATCTTCTGCTACAACCACTG-3'	584	62	60
$GABA_A\alpha2$	798 1380	F:5'-TCTCTCCCAAGTGTCATTCTGGCTG-3' R:5':-GCCCAAAAGTAACCAAGTCTA-3'	583	58	60
$GABA_A\alpha3$	217 953	F:5'-CGGCTTTTGGATGGCTATG-3' R:5'-ATGGTGAGAACAGTGGTGACA-3'	737	60	90
$GABA_A\alpha4$	1045 1594	F:5'-CAGAAAGCCAAAAAGAAGATA-3' R:5'-TAAATGCTCCAAATGTGACTG-3'	550	58	90
$GABA_A\alpha5$	164 723	F:5'-GACTCTTGGATGGCTATGAC-3' R:5'-TGTGCTGGTGCTGATGTTCTC-3'	560	58	90
$GABA_A\alpha6$	52 419	F:5'-CAAGCTCAACTTGAAGATGAAGG-3' R:5'-TCCATCCATAGGGAAGTTAACC-3'	420	58	60
$GABA_A\beta1$	581 1386	F:5'-ATGGAGGAGAGGGAGCAGTAA-3' R:5'-AGAAAAGGTGATGGGGAAGAA-3'	806	55	90
$GABA_A\beta2$	856 1154	F:5'-ACCACAATCAACACCCACCT-3' R:5'-CCCATTACTGCTTCGGATGT-3'	299	58	60
$GABA_A\beta3$	934 1270	F:5'-GGCTGCTTTGTCTTTGTATTC-3' R:5'-TGTGCGGGATGCTTCTGTCTC-3'	337	55	60
$GABA_A\gamma1$	1077 1369	F:5'-CAACAATAAAGGAAAAACCACCAGA-3' R:5'-CCAGATTGAACAAGGCAAAAGC-3'	293	58	60
$GABA_A\gamma2$	553 964	F:5'-ATTGATGCTGAGTGCCAGTTGC-3' R:5'-TGGCTATGGTGCTTAAAGTTGTC-3'	412	60	60
$GABA_A\gamma3$	646 870	F:5'-TGGCGGCTCTATCAGTTTG-3' R:5'-GATGCCTAATGTTGTTCTTGC-3'	225	60	60
$GABA_A\delta$	1079 1532	F:5'-TCGTCCTTTTCTCCCTCTCAG-3' R:5'-AGCCCATCCTGTTCCATCTA-3'	454	58	60
$GABA_BR1$	859 1183	F:5'-ACGCATCCATCCGCCACAC-3' R:5'-AACCAGTTGTCAGCATACCACCC-3'	347	58	60
$GABA_BR2$	2171 2400	F:5'-CCCTGGTCATCATCTTCTGTAG-3' R:5'-CTTCATTCGTAGGCGGTGG-3'	230	58	60
$GABA_C\rho1$	25 751	F:5'-GAATCTATGTTGGCTGTCCAGA-3' R:5'-TGGTGTGGAATTCTTGAAT GAG-3'	775	60	90
GAT1	147 845	F:5'-ACGCTTCGACTTCCTCATGTCCTGC-3' R:5'-GAATCAGACAGCTTTCGGAAGTTG-3'	699	62	90
GAT2	294 606	F:5'-CTGTGGCATCCCAGTGTTC-3' R:5'-GACAGGCGAGGTAAAGTTCTC-3'	313	60	60
GAT3	517 1049	F:5'-AATGTGACCTCCGAGAATGC-3' R:5'-ACCTCAGATATGGGCACACC-3'	533	62	90
GAT4	1130 1864	F:5'-ACCCCAAGGCTGTCACTATG-3' R:5'-CTGTGATGGCAGAGATGGTG-3'	734	62	90
VGAT	31 541	F:5'-TTCTGTCCTTTTCTCCCGCCCCGCCGCC-3' R:5'-GCACCACCTCCCCGTCTTCGTTCTCCTC-3'	572	68	180
αACrys	234 515	F:5'-CCAGAGGCTCCTGTCTGACTC-3' R:5'-TGAGAAAGTGGGAAGGAAACAG-3'	749	53	60

Table 1. Sequences of primers and conditions used in the semi-quantitative RT-PCR analyses. Gene-specific primers were designed for this study according to the published sequences as appeared in Ensembl Genebank (www.ensembl.org). Primer pairs for all studied genes were selected from different exons. The positions are relative to the ATG codons of the corresponding cDNAs. F: forward, R: reverse

was amplified under identical conditions as control (-RT). For quantification, β-actin was co-amplified with the target gene for 20 cycles. When the target gene(s) and the β-actin were separately amplified, the same master mix containing the first strand with the corresponding primers was used. Primer pairs for all studied genes were selected from different exons. Sequences, annealing temperatures and extension times for each primer pair are listed in Table 1. RT-PCR amplification of the αACrys-GAD67-GFP transgene was analyzed by using a forward primer toward the 3' end of the mouse GAD67 cDNA, 5'-GGG GAC AAG GCG ATT CAG T-3', and a reverse GFP primer, 5'- TCA GGG TGG TCA CGA GGG T-3', (see Fig. 9) to give a 605 bp band (conditions: anneling 60 sec at 60°C, extension 90 sec at 72°C). PCR products were separated on 1.5% agarose gels run in TBE buffer (0.089M Tris, 0.089M boric acid and 0.002M EDTA) and bands were visualized with ethidium bromide. For quantification, RT-PCR reactions were performed in triplicate for each gene using RNA from two independent tissue preparations. Quantification of data obtained by densitometry of gel images was carried out using the public domain NIH Image program (free software developed at the U.S. National Institutes of Health available at http://rsb.info.nih.gov/nih-image). For each experimental group β-actin densities were normalized to the maximal actin expression level. The intensities of the PCR bands for the studied genes were then normalized to the corresponding equalized β-actin densities. Relative expression levels for Dlx and GAD transcripts were represented by using a gray scale or plotted in a histogram.

6. Western blotting

Lens protein extracts (15%) were prepared by homogenizing freshly isolated lenses in SB buffer (1mM AET (aminoethylisothiouronium bromide), 0.2 mM PLP (pyridoxal phosphate), 0.1 mM PMSF (phenylmethylsulfonyl fluoride), 0.2 mM EDTA, 1mM benzamidine, 20 µl trypsin-inhibitor (leupeptin-2 µg/ml, pepstetin-2 µg/ml)) containing 1 mM EGTA (ethylene glycol tetraacetic acid) and protease inhibitors (Complete, Roche Applied Sciences). The protein content of each sample was determined (Bradford, 1976). The extracts were quickly adjusted to 1x Laemmli sample buffer by adding 2x Laemmli sample buffer (0. 125M Tris-Cl, pH 6.8, 4 % SDS, 20% (v/v) glycerol, 0.2 M dithiothreitol, 0.02% bromphenol blue) immediately boiled to avoid degradation and centrifuged to remove insoluble material. 30 µg of protein samples were run on 10% SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis), blotted and stained as previously described (Katarova et al., 1990). GAD forms were identified as 30-31 (corresponding to GAD25), 44, 65 and 67 kDa bands, respectively, using anti-pan GAD rabbit serum # 6799.

7. Tissue preparation for immunohistochemistry and in situ hybridization

Pregnant mice at various gestational days were killed by cervical dislocation and embryos were recovered by Caesarean section. Whole embryos or heads were fixed in 4% (w/v) paraformaldehyde (PFA) in PBS for 1 hr at room temperature (RT). For GABA detection, the tissue was fixed in 4% PFA-0.1% glutaraldehyde-PBS overnight at 4°C. The embryonic tissue was immersed in 20% sucrose- PBS solution at 4°C until it sunk, then embedded in Cryo-gel (Instrumedics, Inc.), frozen in dry ice and sectioned at 25 µm on a cryostat (Microm HM550).

Postnatal eyes were removed with curved forceps, placing them behind the globe and gently pulling forward. The eyes were placed in a beaker containing 20 ml ice-cold 4% (w/v) PFA in PBT (PBS containing 0.1% Tween-20) and fixed in a microwave (MW) oven. This method ensures rapid and deep penetration of the fixative inside the lens and provides good ultrastructural preservation. The optimal conditions of MW exposure for the P7 eyes was 2x10 sec; for P14 eyes: 3x8 sec; for P30 eyes: 3x10 sec, respectively, at 25-30°C. The temperature of the fixative was maintained by cooling in ice. Eyes were post-fixed in 4% (w/v) PFA in PBT up to 1 hr at RT, and then subsequently immersed in ascending grades of sucrose- PBS solution at 4°C, embedded in Cryo-gel, sectioned at either 16 or 25 µm and collected on gelatin-coated glass slides.

Tissue for in situ hybridization was fixed and prepared for cryostat sectioning in DEPC-treated solutions. The embryos and eyes were sectioned at 20 µm and mounted on poly-L-lysine coated slides.

For Hemalaun-Eosin staining several sections were rehydrated through a graded series of ethanols and stained with Hemalaun (Fluka Chemie) for 2 minutes, and Eosin (Reanal ZRt) for 1.5 min. Sections were then dehydrated, cleared and cover-slipped with Entellan (Merck KGaA).

8. Immunohistochemistry

8. 1. GAD and GABA immunohistochemistry

For GAD staining, sections were briefly dried, washed in PBS and dehydrated in graded methanol in PBS for 15 min/step, stored in 100% methanol overnight at -20°C and finally rehydrated through descending methanol series into PBS. Subsequently, sections were incubated in 1% H_2O_2 in PBS for 30 min, washed 3x10 min with PBT and blocked for 2 hrs in 0.5% Blocking Reagent (Roche Applied Sciences) in PBS. The sections were then incubated overnight at 4°C with rabbit anti-GAD serum #6799 (Katarova et. al., 1990) at a dilution of 1:500 or rabbit anti-GAD65 serum N65 at 1:500 (a kind gift from Pietro de

Camilli, Yale University; Solimena et al., 1993) in 1% bovine serum albumin (BSA, Sigma-Aldrich CO) in PBT. Sections were washed 3x10 min at RT with PBT, then incubated for 2 hrs with a biotinylated anti-rabbit antibody (1: 500 in PBT, Vector Laboratories), followed by 2x10 min PBT, 1x10 min PBS washes. Sections were subsequently incubated with ExtrAvidin-HRP (Sigma-Aldrich Co.) diluted 1:500 in 1% BSA-PBS) for 90 min at RT. Finally sections were reacted with 0.3 mg/ml diaminobenzidine (DAB, Sigma-Aldrich CO) in TBS (Tris-buffered saline, 50 mM Tris-Cl, pH 7.4, 0.15 M NaCl) in the presence of 0.001% H_2O_2.

GABA immunohistochemistry was performed essentially as described (Katarova et al., 2000). Sections were incubated with affinity-purified anti-GABA rabbit polyclonal serum (Sigma-Aldrich Co.) at a dilution 1:5000 overnight at 4°C in 1% PBS with 1% BSA and 0.1% Tween-20 (BSA-BPT), washed in PBT, and incubated with the ABC Elite Kit (Vector Laboratories) reacted with diaminobenzidine. Cryostat sections from GAD65_3e/gfp5.5 #30 transgenic mice were cover-slipped with Mowiol-488 (Calbiochem-Merck Biosciences) as recommended by the supplier. Sections were examined under Zeiss Axioscop-2 microscope equipped with AxioCam HRc digital camera (Carl Zeiss AG) using AxioVision 4.2-4.6 software.

8. 2. Immunofluorescent labeling

For immunofluorescent detection of GAD, GABA, DLX2, $GABA_A\beta3$ and $GABA_BRII$ receptor subunit, VGAT, GAT-1, GAT-3, Ki-67, N-cadherin, Pan-cadherin in wild-type and αACrys-GAD67 transgenic lenses, slides with serial cryostat sections were dried, washed with TBS (50mM Tris-HCl, 150mM NaCl, pH 7.5) for 5 min. They were subsequently blocked in 1% BSA, 0.02% saponin (Sigma-Aldrich Co.) in TBS for 2 hours at room temperature. The sections were incubated with primary antibody (rabbit anti-GAD serum #6799 (1:500), rabbit anti-GABA (Sigma-Aldrich Co., 1:5000), rabbit anti-DLX2 (Chemicon, 1:400), rabbit anti-$GABA_A\beta3$ (Novus Biologicals, 1:1000), rabbit anti-$GABA_BR2$ (BD Biosciences, 1:500), guinea pig anti-VGAT (Calbiochem, 1:5000), guinea pig anti-mouse GAT1 (gift from N. Nelson, 1:1000), rabbit anti-GAT3 (Abcam, 1:1000), rabbit anti-Ki-67 (Abcam, 1:1000), mouse anti-N-cadherin (BD Transduction Laboratories, 1:1000), mouse anti-Pan-cadherin (Sigma-Aldrich Co., 1:1000)) diluted in 1% BSA, 0.005% saponin in TBS, overnight at 4^oC. After washing with TBS containing 0.005% saponin, the sections were incubated for two hours with biotinylated secondary antibody (1:500, Vector Laboratories) in 1% BSA, 0.005% saponin in TBS, followed by three washes with TBS, 0.005% saponin. For visualization, Streptavidin-conjugated Cy3 (1:2000, Jackson Res.) or Streptavidin-conjugated

Oregon Green-488 (1:200, Molecular Probes) antibody was applied for two hours at room temperature. Sections were washed with TBS, and mounted with Mowiol (Calbiochem) supplemented with 1,4-diazabicyclo-[2,2,2]-octane (DABCO, 2,5% (w/v), Sigma-Aldrich Co.).

Specimens with immunofluorescen labeling were examined under Zeiss Axioscop-2 microscope supplied with AxioCam HRc using AxioVision 4.6 software or confocal laser scanning microscope Zeiss LSM 510 META and Olympus FV500.

For cell proliferation counts, Ki-67 immunopositive cells were quantified on 20µm serial sections through the lens epithelium (7 central lens sections from 4 different eyes).

9. Non-radioactive *in situ* hybridization

9. 1. Probe synthesis

GAD67 antisense riboprobe was used to detect GAD67 mRNA in wild type and transgenic lenses. In CrysGAD67 lenses transgene derived mRNA was detected by using SV40 riboprobe. Sense counterparts of the antisense probes were used as controls for *in situ* hybridization. The GAD67 probes were synthesized on DNA templates that were prepared according to a protocol described in Katarova et al., 2000. The SV40 polyadenylation sequence from the CPV2 plasmid was subcloned into pBluescript II SK+ (Stratagen), linearized with *EcoRI* and *XbaI* and used to synthesize antisense and sense riboprobes using T3 and T7 polymerases. DIG-α-UTP was used to generate non-radioactive dioxigenin labeled RNA probes as recommended by the supplier (Promega).

9. 2. Hybridization with DIG-UTP-labeled probes

Hybridization and washings were performed as follows: The sections were washed in PBS, treated with 1mg/ml proteinase K for 10 minutes at 37°C, then postfixed for 20 minutes in 4% PAF, washed in PBS, and treated in 0.1M triethanolamine buffer, pH 8.0, containing 0.25% (v/v) acetic anhydride (Sigma-Aldrich Co.) for 2x5 minutes at room temperature. Prehybridization was performed in 50% formamide in 5xSSC (Sodium Chloride Sodium Citrate buffer: 0.75 M NaCl, 0.075 M, Na3citrate·2H2O pH 7) for 1 hour at room temperature. The hybridization solution contained 0.5–1.0 mg/ml DIG-UTP labeled cRNA probes in 50% formamide, 5xSSC, 1% sodium dodecyl sulfate, 50 mg/ml yeast tRNA, and 50 mg/ml heparin. The solution was heated to 90–95°C, applied to the sections at 60°C, and covered with a piece of parafilm. Hybridization was performed in a humid chamber at 55°C overnight. Post-hybridization washing was performed in 2xSSC, 50% formamide at 50°C, followed by RNaseA digestion and by a further high stringency wash (0.1xSSC). Anti-DIG

antibody conjugated to human placental alkaline phosphatase (Boehringer Mannheim) was applied at 1:2000 dilution in PBT containing 1% normal horse serum, and sections were incubated O/N at 4 ^{o}C. After washing with PBT containing 2mM levamisol the sections were incubated in alkaline phosphatase (AP) buffer (100mM NaCl; 100mM Tris, pH 9.5; 50mM $MgCl_2$, and 1.1% Tween-20 with 2mM levamisol). Color detection was performed as described by Wilkinson and Nieto (1993).

10. GABA measurement by high performance liquid chromatography

Frozen lens tissue (two-E15.5-E16.5, one-E17.5 lens) was homogenized in 50 µl 0.2 M ice-cold perchloric acid, cleared by centrifugation (3510xg for 10 min at 4^{o}C) and neutralized by addition of 2M KOH (25:7 ratio). The precipitate was removed by centrifugation as above, and the supernatant was kept at -20^{o}C until analysis. The pellet was saved for for measuring protein concentration according to Lowry et al., using bovine serum albumin as standard. Derivatized 1-(alkylthio)-2-alkylisoindol amino acid adducts were separated on a 5 µm Discovery HS C-18 (150x4.6 mm) analytical column that was equilibrated with 11.25% methanol-acetonitrile (3.5:1 v/v) in 0.01 M PBS, pH 7.2. The mobile phase was 22.2% acetonitrile in methanol and the profile of the gradient was as described by Mally et al. (1996). 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: **(A * f * B)/(C * D * E)** (**A**: Area of GABA; **B**: Sample volume; **C**: Injection volume; **D**: Response factor of 1 nmol GABA standard; **E**: Protein content of sample; **f**: factor of Internal Standard (IS area in calibration/IS area in actual)). The data are expressed per µg protein.

11. Primary lens epithelial cell culture

Mouse P0 lenses were dissected from deeply anaesthetized newborn (P0) mice in ice-cold PBS under a dissecting microscope. Lenses were cleaned of cilliary epithelium and retina with fine forceps and collected in groups of ten in ice-cold cell-culture grade Tyrode' salts (Sigma-Aldrich). Subsequently lenses were transferred into 500 µl 0.02% EDTA solution in 0.5 mM DPBS (Dulbecco's Phosphate Buffered Saline Sigma-Aldrich) at 37°C for 10 min. One milliliter of Medium 199 (Invitrogen) supplemented with 10% fetal bovine serum (FBS, EuroClone) and antibiotic was added and the suspension was triturated 10 times. Cells were centrifuged at 200xg for 2 min, resuspended in fresh Medium 199-10% fetal calf serum (FCS) and plated on matrigel-coated coverslips (BD Biosciences, 0.4 mg/ml in PBS) at the equivalent of one lens/cm^2. Primary lens epithelial cell cultures were incubated at 37°C at

5%CO_2/95%O_2. Medium was replaced every 3-4 days. Two week-old cultures comprised of both single lens epithelial cells, epithelial monolayers and lentoids of different sizes were used for immunocytochemistry and Ca^{2+} imaging.

12. Immunocytochemistry

Primary LEC cultures grown on coverslips were fixed in 4% PFA in PBS (4% PFA, 0.1% glutaraldehyde, PBS for GABA detection) for 20 min at RT and washed 3x5 min in PBS. Cells were permealized in PBS containing 0.2% Triton X-100 (Sigma-Aldrich) for 5 min at RT, blocked in 0.5% blocking reagent (Roche Applied Sciences) in PBS for 1 hr at RT and subsequently incubated overnight at 4°C with primary antibodies in 1% BSA, PBT (0.05% TWEEN® 20 – Sigma-Aldrich, in PBS) as follows: goat anti-αA-crystallin (Santa Cruz Biotechnology, 1:500), rabbit anti-αB-crystallin (Biomol International Inc., 1:500), rabbit anti-GAD serum #6799, at 1:500, rabbit anti-$GABA_A$β3 (Novus Biologicals, 1:1000), rabbit anti-$GABA_B$R2 (BD Biosciences, 1:500), guinea pig anti-VGAT (Calbiochem, 1:5000), guinea pig anti-mouse GAT1 (gift from N. Nelson, 1:1000), rabbit anti-GAT3 (Abcam, 1:1000), mouse anti-uvomoruline (E-cedherin, Sigma-Aldrich Co., 1:1000), mouse anti-N-cadherin (BD Transduction Laboratories, 1:1000). After 3x10 min washes in PBT, anti-mouse (guinea pig)-Alexa Fluor® 594, anti-goat (anti-rabbit, anti-mouse)-Alexa Fluor® 488-conjugated secondary antibodies (Invitrogen, all at 1:500 dilution) in 1% BSA-PBT were applied for 1 hr at RT. Cells were washed 3x10 min in PBT, 1xPBS, coverslipped in Mowiol (Calbiochem) and visualized under Zeiss Axioscop-2 or Zeiss LSM 510 META.

13. $[Ca^2]_i$ imaging studies

Freshly isolated lenses from neonatal mice were washed in Hepes-buffered saline solution (HBSS) containing (in mM): 137 NaCl, 5 KCl, 20 HEPES, 10 glucose, 1.4 $CaCl_2$, 3 $NaHCO_3$, 0.6 Na_2HPO_4, 0.4 KH_2PO_4 at pH 7.4. Intact lenses were loaded for 30 min at 37°C with 5 μM Fluo-4/AM in the presence of 0.1% pluronic acid F-127 (both from Molecular Probes). Subsequently the lenses were secured by means of a mesh to the bottom of a plastic perfusion chamber mounted on a laser-scanning microscope (LSM, Olympus FV-500). Recordings were at 488/515 nm excitation/emission. Image acquisition was set at one image/1.8 sec. Cells were superfused with HBSS buffer at a rate of 2 ml/min using a peristaltic pump (Gilson) initially for 2 min with HBSS to wash out excess dye. Additional 2 min perfusion with HBSS was performed to obtain a steady baseline, then 1 mM GABA in HBSS was applied for 2 min, followed by 2 min HBSS and finally HBSS-20 mM KCl. Analysis of fluorescence intensity was performed using the imaging software of the Olympus

FV500 (FluoView^{TM}).

Primary LEC cultures were loaded with Fluo-4/AM (2.5 μM Fluo-4/AM, 0.1% pluronic acid) in growth medium at 37°C for 45 min, in a humidified atmosphere of 5% CO_2/95%/O_2. Subsequently the medium was changed and de-esterification of the dye was allowed to proceed for another 15 min. The glass coverslips with attached cells were placed in a POC-R type submerged chamber maintained at 24°C (PeCon). In each experiment cells were perfused for 2 min at 2 ml/min with artificial aqueous humor (AAH) of the following composition (in mM): NaCl, 130; KCl, 5; $CaCl_2$, 1; $MgCl_2$, 0.5; D-glucose, 5; $NaHCO_3$, 5; HEPES, 10; pH 7.25 to obtain a steady baseline. GABA (1 mM), muscimol (100 μM) or baclofen (20 μM), all from Sigma-Aldrich were applied for 2 min, followed by a 10 min perfusion with AAH.

Whenever $GABA_A$ or $GABA_B$ receptor antagonists were used, cells were initially perfused for 2 min with the antagonist: 20 μM of bicuculline (Sigma-Aldrich; a $GABA_AR$ antagonist) or 20 μM of CGP55845 (Tocris; $GABA_BR$ antagonist) followed by 2 min with 100 μM muscimol + 20 μM bicuculline or 20 μM baclofen + 20 μM CGP55845, respectively. Perfusion was continued for another 10 min with AAH then cells were perfused for 2 min with AAH containing only the agonist (muscimol or baclofen, respectively). Cultures were observed under Zeiss LSM 510 META (Carl Zeiss) laser confocal scanning microscope and scanned every second. All settings of the laser, optical filter and microscope as well as data acquisition were controlled by LSM 510 META software. Excitation was at 488 nm and emitted light was read at 515 nm. Analyses were performed at the single-cell level and expressed as relative fluorescence intensity.

14. Statistical analysis

Statistics were performed using Graph Pad Software Prism 5.0 (GraphPad Software Inc. CA). 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. All the figures shown in this dissertation were obtained from at least three independent experiments with a similar pattern.

To find possible co-regulation among the genes involved in the GABA-signaling pathway, we performed nonlinear pair-wise correlation tests of relative gene expressions spanning all studied developmental stages (Kotlyar et al., 2002). Data were analyzed with Spearman correlation test using Graph Pad Prism 5.0 software. In all cases, $p<0.05$ was considered significant.

V. Results

1. Expression of Dlx and GAD genes in the developing lens at RNA level

1. 1. Semi-quantitative RT-PCR analysis of different GAD and Dlx transcripts

Semi-quantitative RT-PCR analysis using first-strand cDNA from embryonic (E14.5, E15.5, E16.5, E17.5) and postnatal (P0, P7, P14, P30) mouse lenses was performed to study temporal expression pattern of GAD genes during lens development. Embryonic stage E14.5, when the lens is composed of epithelium and predominantly primary lens fibers, was chosen as a starting point for technical reasons (see Materials and Methods).

In this study, for amplification of different GAD transcripts, we used PCR primer-pairs specific for embryonic GADs, and for adult GAD67, GAD65 isoforms. RT-PCR amlification of adult GAD67 was performed using a forward primer spanning the exon 6-8 border region (Table 1), thus precluding amplification of exon 7A/B-containing embryonic cDNAs. For joint amplification of the embryonic GAD transcripts I-86 and I-80, (EGAD) reverse primer was derived from embryonic exon7A/B. We found that all three types of GAD mRNAs were already present in E14.5 lenses, but had distinct expression profiles during later stages of lens development. The embryonic GAD transcripts (EGAD) were already expressed at a considerably high level at E14.5 then slightly increased in intensity with a maximum level of expression at E16.5, declining sharply thereafter and were barely detectable at postnatal stages (Fig. 10). GAD67 was expressed at a constant low level until birth (P0), and then gradually increased, peaking postnatally at P14-P30 (Fig. 10A, B). In contrast, GAD65 mRNA was highly expressed at E14.5, peaked at E15.5, and then decreased sharply from E17.5 onwards, being virtually undetectable after P14 (Fig. 10A and B).

We also examined the presence of Dlx2 and Dlx5, two transcription factors that overlap with the expression of GAD in spatially-restricted domains in the developing forebrain. Our semi-quantitative RT-PCR results showed that similarly to the developing forebrain, Dlx2 expression in the lens was induced before Dlx5. As shown in Fig. 10, Dlx2 mRNA levels were high and remained almost unchanged throughout E14.5-E16.5, after which Dlx2 expression declined and was undetectable in P7 and older lenses (Fig. 10A, B). Dlx5 mRNA, on the other hand, was barely detectable at E14.5 then gradually increased between E15.5 and P0, reaching a maximum at around birth (E17.5-P0). In the postnatal lens Dlx5 was steeply down-regulated and was not detected beyond P30 (Fig. 10A, B). It should be noted that both adult and embryonic GAD forms (data not shown) and Dlx1/2 are abundantly expressed in the retina during all developmental stages including the adult, thus the lens-specific profile cannot be attributed to contamination from retinal tissue.

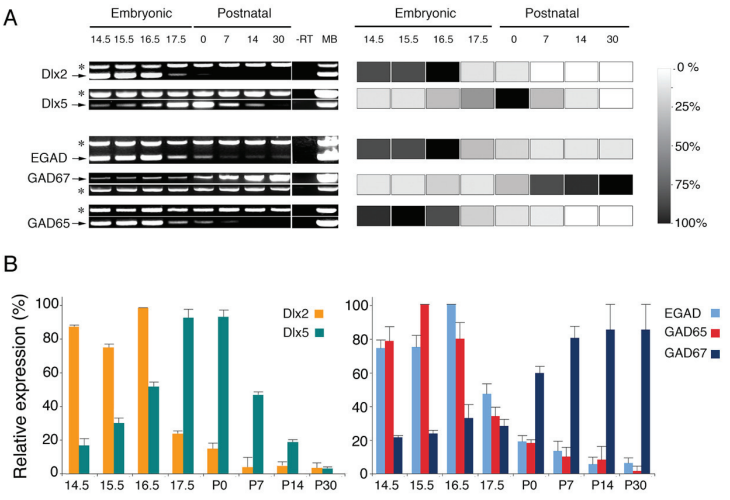

Fig.. 10. Temporal expression patterns of Dlx2, Dlx5 and GAD gene family in developing mouse lens. I. Representative RT-PCR amplifications of Dlx2 and Dlx5 and different GAD (EGAD, GAD67 and GAD65) transcripts using cDNA synthesized from total RNA preparations of E14.5-P30 lenses and adult mouse brain (MB). For quantification, β-actin (*) was co-amplified with the target genes. Expression levels were normalized to β-actin, and represented on a grey scale as % of max level. **II.** Histograms (means ± SEM) of relative expression level of each gene calculated from three independent experiments normalized to the maximum expression level of co-amplified β-actin (100%).

1. 2. GAD67 expression in the developing lens as revealed by in situ hybridization

For in situ hybridization studies, we used such a GAD67 antisense probe that recognizes both embryonic and adult messages encoded by the GAD67 gene. However based on the RT-PCR results most, if not all GAD67-related labeling during embryonic stages is due to EGAD and only adult GAD67 is expressed at P30 (Fig. 10). At E15.5 GAD67-related expression was localized in both primary and secondary fiber cells with strongest labeling in the epithelial cells and cortical fiber cells (Fig. 11B, C). At birth, expression was relatively strong and limited to the lens epithelium and cortical secondary fiber cells (Fig. 11E, F). From P0 GAD expression was absent from the fiber cells in deeper regions of the lens (Fig. 11E, H). At P30 increased labeling was observed in the lens epithelium and elongating secondary fibers at the equatorial region (Fig11H, I). No specific hybridization was seen in any of the sense controls (Fig. 11A, D, G).

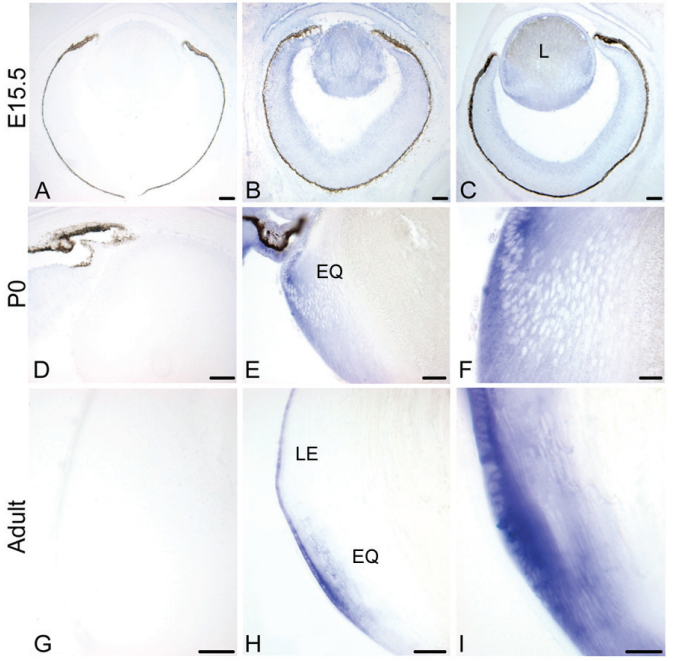

Fig. 11. Expression pattern of GAD67-related mRNAs (EGAD, GAD67) during lens development. Hybridization of dioxigenin (DIG)-labeled GAD67 probe on coronal cryosections from E15.5 (A-C) mouse embryos and P0 (D-F), P30 (G-I) postnatal eyes. The sections shown in F and I are higher magnification views of the boxed areas shown in E and H, indicating GAD expressing cells in the equatorial region. No specific hybridization was seen on sense control sections (A, D, G). Abbreviations: EQ-equatorial region; L- lens; R-retina. Scale bars: A-C 100 μm; D, E, G, H 50 μm; F, I 50 μm.

2. Temporal regulation of GAD proteins and GABA in the developing lens

We performed Western blot and high performance liquid chromatography (HPLC) analysis to study the synthesis of GAD proteins and determine GABA content through E15.5 embryonic-P30 postnatal stages. To this end, protein homogenates from E15.5-P30 lenses were run on SDS-PAGE gels, blotted at conditions optimized for each isoform and stained with a pan-GAD rabbit serum #6799. β-actin was used as an internal control to monitor the amount of protein applied on the gel. P30 retina expresses high levels of all GAD isoforms (Haverkamp and Wassle, 2000; Dkhissi et al., 2001; de Melo et al., 2003), its homogenate was run in parallel as a positive control (Fig. 12A: P30-R). In agreement with the RT-PCR results, GAD65 was more abundant at earlier stages (E15.5-E17.5) peaking at E17.5, thereafter its level quickly dropped and was not detected at P30. In contrast, GAD67 appeared first as a faint band at P0, slightly increasing until P30 (Fig. 12. I. A). EGAD

(GAD25 and GAD44) showed a transient temporal expression pattern similar to that characteristic for the embryonic nervous system: it was low at the earliest stages (E15.5), peaked at E17.5, the stage of most intensive primary fiber cell terminal differentiation, gradually decreased until P14 and was undetectable beyond this stage (Fig. 12. I. A).

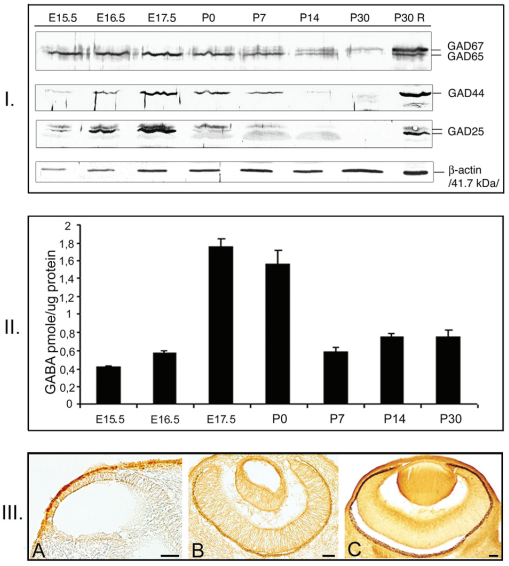

Fig. 12. GAD proteins and GABA content in the developing mouse lens. I: Western blot analysis of GAD forms in the eye lens at stages E15-P30: protein homogenates from lenses isolated from the different stage embryonic lenses next to P30 retina (P30R) were run on 10% SDS-PAGE, blotted and stained with an anti-GAD rabbit serum #6799. β-actin was stained as control. The embryonic stages are characterized by a robust expression of GAD65 and less embryonic GADs (EGAD- GAD25 and GAD44). GAD65 and EGADs are transiently expressed with a peak at E17.5. Note that the GAD67-specific band appears around P0 and becomes more pronounced during subsequent stages although the level of up-regulation is not as high as observed by RT-PCR. **II:** HPLC analysis of GABA content in the developing mouse lens: GABA concentration was measured in E15.5-P30 lens homogenates. Bars represent means of three measurements ±SEM. **III.** Expression of GABA in the developing mouse lens. A-C: coronal sections from different stage embryos (A-E9.5, B-E11.5, C-E13.5). Scale bar: 100 μm.

To determine if GABA levels changed with GAD mRNA and protein levels during differentiation, a HPLC was used to determine relative GABA content. The GABA content of the lens at different developmental stages showed a clear correlation with the temporal profile of GADs: it was low at earlier stages, increased considerably and peaked at E17-P0, then leveled to 30% of P0 value at the postnatal stages (Fig. 12. II. B).

In E9.5-E13.5 embryos GABA was detected by immunohistochemistry for technical reasons (see Materials and Methods). GABA had a similar spatial distribution in the developing lens as GAD, consistent with GAD being enzymaticaly active. Strong GABA staining was visible in the lens placode (Fig. 12. III. A), later in the lens vesicle (Fig. 12. III. B), in the lens epithelium and primary fiber cells at E13.5 (Fig. 12. III. C).

3. Spatial segregation of GAD forms during lens development

To detect the GAD67 gene-encoded GAD isoforms we used an anti-GAD antibody (serum #6799), which was raised against the recombinant mouse GAD67, but also recognizes the truncated GAD25 and GAD44, and to a lesser extent the GAD65 on Western blots. This

antibody does not bind to GAD65 on tissue sections under the conditions used in this study (Z. Katarova, unpublished results). To reveal the spatial expression pattern of GAD65, we used the GAD65-specific antibody N-65.

Immunolabeling of embryonic and postnatal eye sections from E9.5 to P30 using anti-GAD67 serum #6799 is demonstrated in Fig. 10 and 11. Based on our RT-PCR and Western blot results we could conclude that the pattern obtained with serum #6799 reflects the expression of EGAD (GAD25 and GAD44) in embryonic and predominantly that of GAD67 in the postnatal lens.

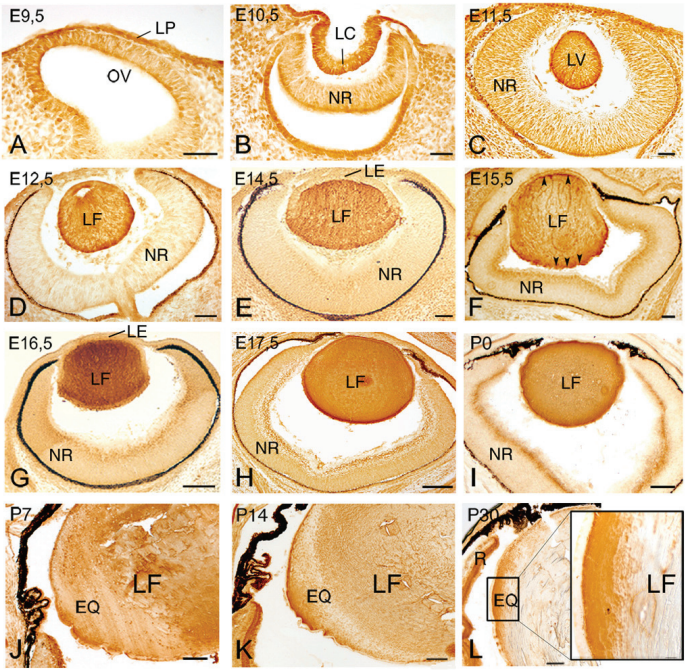

Fig.. 13. Expression patterns of GAD67-related proteins during lens development. Coronal sections from E9.5-P30 eyes were stained with anti-GAD serum #6799. Based on the RT-PCR and Western blot results most, if not all GAD67-related immunoreactivity during embryonic stages is due to GAD25 and GAD44; only adult GAD67 is expressed beyond P7. GAD67-related immunoreactivity was detected from the lens placode stage, thereafter was localized to the lens pit, primary fibers and central epithelium. **EQ**-equator**; gcl-**ganglion cell layer; **inbl-**inner neuroblastic layer of retina; **ipl-**inner plexiform layer of retina; **LC**-lens cup; **LF**-lens fibers; **LP**-lens placode; **LV**-lens vesicle; **NR**-neural retina**; OV**-optic vesicle. Arrowheads in D and F point to the high accumulation of immunostaining product at the anterior/posterior lens poles. The boxed area in **L** includes elongating fibers at the equatorial region, prominently labeled for GAD67. Scale bars: A-J 100 μm; K-50 μm.

GAD67 staining was already detected in the lens placode, an area of a thickened surface ectoderm and the underlying optic vesicle at E9.5 (Fig. 13A). At E10.5-E11.5,

uniform immunolabeling was observed in the lens cup and lens vesicle, respectively (Fig. 13B, C). At E12.5, strong staining was observed in both the posterior wall, containing elongating primary fibers, and epithelial cells at the anterior pole, which remain undifferentiated and retain their proliferation capacity (Fig. 13D).

By E13, the lens vesicle lumen is obliterated by primary fibers. Thereafter, mitotically active cells near the equatorial region (termed germinative epithelium) divide and migrate towards the equator, where they elongate and differentiate into secondary lens fiber cells, a process continuing throughout life. From E14.5 on, the GAD67–related proteins were localized in both primary and secondary fiber cells with strongest labeling at E16.5-E17.5 (Fig. 13). This pattern was preserved until birth.

In postnatal lenses, a gradual change could be followed from moderately strong intensity (P0) to complete absence of staining (P30) of the lens core (Fig. 13; P0-P30) concomitant with increase in immunolabeling of elongating secondary fibers at the equatorial region (EQ in P7-P30).

Staining in the retina in all cases was identical to the previously reported pattern and was monitored as control (Haverkamp and Wassle, 2000; Dkhissi et al., 2001; de Melo et al., 2003).

Prominent GAD expression in embryonic lenses was observed at the apical and basal cell poles of the elongating lens fibers, which indicates that GADs (and GABA-not shown) are selectively targeted to these sites (Fig. 13, D, F; Fig. 14, A-E). The highest intensity of GAD67 immunlabeling was observed at the anterior and posterior lens poles of E16.5 lenses, at the sites of contact between (primary) lens fibers of opposite sides during formation of the prospective lens sutures (Fig. 14C-E).

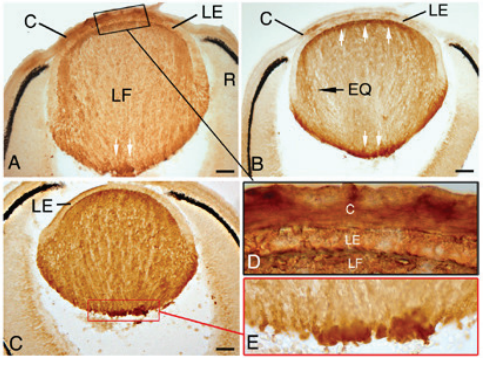

Fig.. 14. Enrichment of GAD67-reated protein expression at elongating fiber tips and attachment sites. E16.5 lenses were immunostained with anti-GAD67 serum #6799 reacting mostly with EGAD at this stage (see Results). Labeling is highly enriched in the tips of elongating primary fibers, the fiber/capsule and fiber/lens epithelium interface (white arrows in **A** and **B**, boxed area in **A** shown enlarged in **D**). The strongest signal was observed near the newly-forming lens sutures (arrows in **B**, enlarged boxed area from posterior lens of **C** shown in **E. C**- cornea**; EQ**- equator; **LE**- lens epithelium; **LF**- lens fibers; **R**- retina. Scale bar: 100 µm.

In addition to fiber cells, conspicuous labeling for GAD was present in the central parts of the lens epithelium and overlying cornea on mid-coronal sections (Fig.14, C and enlarged boxed section of C) that disappeared gradually towards the flanking regions and the adjacent germinative epithelium (Fig. 14D, E). This unique pattern was highly reproducible in E14.5-E16.5 lenses (Fig. 13 and 14).

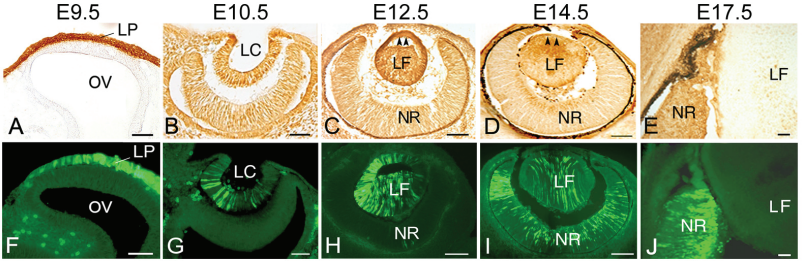

Fig. 15. Expression pattern of GAD65 in the developing mouse lens. **A-E**: coronal sections from different stage embryos stained with anti-GAD65 antibody N-65. **F-J**: sections from GAD65/gfp transgenic embryos expressing GFP under the control of GAD65 promoter. Similar to GAD67, GAD65/gfp fusion protein was localized initially to the lens placode, lens vesicle and later to lens primary fiber cells. GFP expression in the lens disappeared beyond E17.5, but GAD65 immunoreaction was still present at very low levels in the lens core until P7 (data not shown). Arrowheads point to lens epithelium. **inbl:** inner neuroblastic layer of retina (see; **LC**- lens cup**; LF**- lens fibers**; LP**- lens placode; **NR**- neural retina; **OV**- optic vesicle. Scale bar: 100 µm.

The spatial pattern of GAD65 expression in embryonic lenses was revealed by immunostaining with the GAD65-specific N-65 antibody of wild-type E9.5-E17.5 eyes (Fig. 15, A-E) and by GFP expression in GAD65/gfp transgenic eyes of the same age (Fig. 15, F-J).

Strong GAD65 staining was first visible in the thickened surface ectoderm of the lens placode (Fig. 15A) and persisted through the lens pit (E10.5; Fig. 15B) and lens vesicle (E11.5; not shown) stages. At E12.5, strong labeling was detected in the anterior wall (prospective lens epithelium) and elongating cells of posterior wall (prospective primary lens fibers) (Fig. 15C). The core lens fibers were still prominently stained at E14.5 (Fig. 15D), thereafter the intensity was reduced (Fig. 15E). Faint GAD65 expression was still detected in P0 and P7 lens nucleus, but disappeared at later stages. The GFP expression pattern in GAD65/gfp transgenic mouse lenses was indistinguishable from that of GAD65 immunostaining at stages E9.5-E16.5 (Fig. 15F-I and data not shown). Intense GFP labeling was observed at E9.5 in the lens placode (Fig. 15F), in the lens cup at embryonic day 10.5 (Fig. 15G), in the lens epithelium and primary fiber cells at E12.5 (Fig. 15H) and later in primary fiber cells at E14.5-E16.5 (Fig. 15I and data not shown). Interestingly, we failed to detect GFP beyond stage E16.5 in the lenses of five different GAD65/gfp transgenic mouse lines generated in our lab (Fig. 15J and data not shown), although GAD65 protein was clearly

present until P7 (Fig. 15).

4. Expression pattern of DLX2 in the developing lens

Our RT-PCR data clearly indicates that during lens development, Dlx2 shows a temporal expression pattern similar to GAD65 and EGAD (Fig. 10). We used a DLX2-specific antibody to study the cellular distribution of the protein. DLX2 protein was found in the nuclei of lens epithelium and differentiating primary and secondary lens fibers during stages E11.5-P14 (Fig. 16A-F), indicating that similar to developing forebrain, DLX2 co-localizes with GAD (both GAD65 and GAD67) in the lens as well. At E11.5 DLX2 was present with a low expression levels when compared to adjacent dorsal retinal neuroepithelium (Fig. 16A). The intensity of immunostaining was strong during late embryonic stages and the first week postpartum, but declined thereafter, in agreement with the PCR data. Interestingly, DLX2 labeling was absent from nuclei in more advanced stages of degradation found in the lens core at P7**.** It should be noted, that DLX2 shows overlapping pattern of expression with GAD65 and GAD67 also in the retina and cilliary epithelium (Fig. 16 and de Melo et al, 2003).

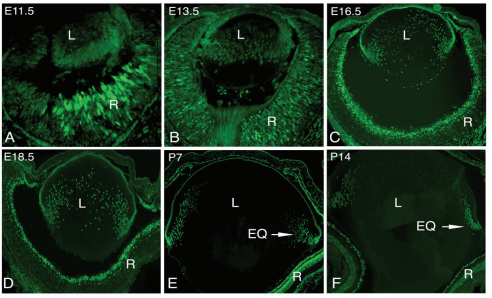

Fig. 16. DLX2 expression labels both epithelium and fiber cells of the developing lens. A-D: Coronal sections from different embryonic stages were immunostained with anti-DLX2 antibody. DLX2 expression in lens was detected as early as E11.5. By E13.5, DLX2 expression was well-established, and the intensity of immunostaining was strong during late embryonic stages (**C**, **D**), as well as at P 7 (**E**). **E-F**: DLX2 expression in the postnatal lens was similar to the pattern found in embryonic stages, with an absence of staining in nuclei of the lens core. Scale bars: 50 μm in **A**, **B**; 100 μm in **C-F**.

5. Expression of GABA receptors and transporters in the developing mouse lens

5.1. RT-PCR analysis reveals that $GABA_A$ and $GABA_B$ receptor subunits and GABA transporters are abundantly expressed in the developing lens

Our study was the first attempt to reveal the presence and characterize the expression profile of the downstream GABA signaling components during mouse lens development. We showed and characterized the temporal expression profile of mRNA encoding 11 different $GABA_A$ receptor subunits (α_{1-4}; β_{1-3}; γ_{1-3} and δ), two $GABA_BR$ subunits, VGAT and GAT1-4, respectively during developmental stages E14.5-P30 obtained by semi-quantitative RT-PCR (Fig.16A). These expression profiles were analyzed and grouped according to developmental stage (embryonic, fetal, neonatal and postnatal; Fig. 17B). Furthermore, applying Spearman's

correlation (Kotlyar et al., 2002) we obtained the correlation coefficient for a number of gene pairs with highly similar profiles (Fig. 17C1-C6).

The functional $GABA_AR$ is a heteropentamer assembled from two α, two β and one γ (or δ, ε, π, θ) subunits (Watanabe et al., 2002; Rudolph et al., 2001). Out of six α subunits (α_{1-6}), we failed to detect α_5 and α_6 at any of the studied stages. α_2 and α_3 subunits were abundantly expressed during embryonic stages (E15.5-P0) in contrast to α_1, which is mostly postnatal. α_4 had a steady elevated expression beyond E16.5 (Fig. 17A). Of the three known β subunits, β_1 displayed low expression throughout all studied stages with a moderate elevation at P0 (Fig. 17A, B). β_3, by far the most abundant subunit at embryonic stages was dramatically reduced after P0, while β_2 was low at E14.5 and transiently upregulated at E16.5-P7. All three γ and the δ subunits were expressed from E14.5 (Fig. 17A,B). γ_1 was predominantly prenatal and almost undetectable beyond P0, while γ2, γ3 and δ were present at all studied stages. The fairly abundant γ_2, and δ were moderately upregulated at E16.5-P0. γ_3 is by far the most abundant of all γ subunits, which contrasts its highly restricted expression in the brain (Wisden et al., 1992; Laurie et al., 1992a, 1992b; Pirker et al., 2000)**.** The high level of expression of γ3 throughout all stages and that of α_4 beyond E16.5 is striking, inasmuch as it is not paralleled by a similar high level of expression of any of the β subunits (Fig. 17A, B) suggesting that the relative levels of active $GABA_AR$ in the postnatal lens may be determined by the availability of $\beta_{2/3}$ subunits.

We failed to detect amplification products of the retina-specific $GABA_CR$ subunits ρ1 and ρ2, which demonstrates that all RT-PCR reactions are strictly lens-specific.

The functional $GABA_B$ receptor is a heterodimer comprised of two subunits - $GABA_BR_1$ harboring the GABA binding site and $GABA_BR2$ responsible for dimer formation (Bettler et al., 2004; Couve et al., 2004). Using specific PCR primers we found, that both transcripts, R1 and R2 are present in the developing lens, but show very different expression profiles (Fig. 17A). $GABA_BR1$ showed a strong and uniform expression at all stages, whereas $GABA_BR2$ was extremely low at most stages with the exception of fetal lens (E15.5-E17.5).

The GABA transporters, both vesicular and membrane transporters, showed abundant expression in the developing lens, with distinct patterns (Fig. 17A). GAT1-3 were low at early stages, after E16.5 GAT2/3 increased, while GAT1 was transiently elevated at E17.5-P0. GAT4 was highly and nearly uniformly expressed throughout all stages. VGAT showed a

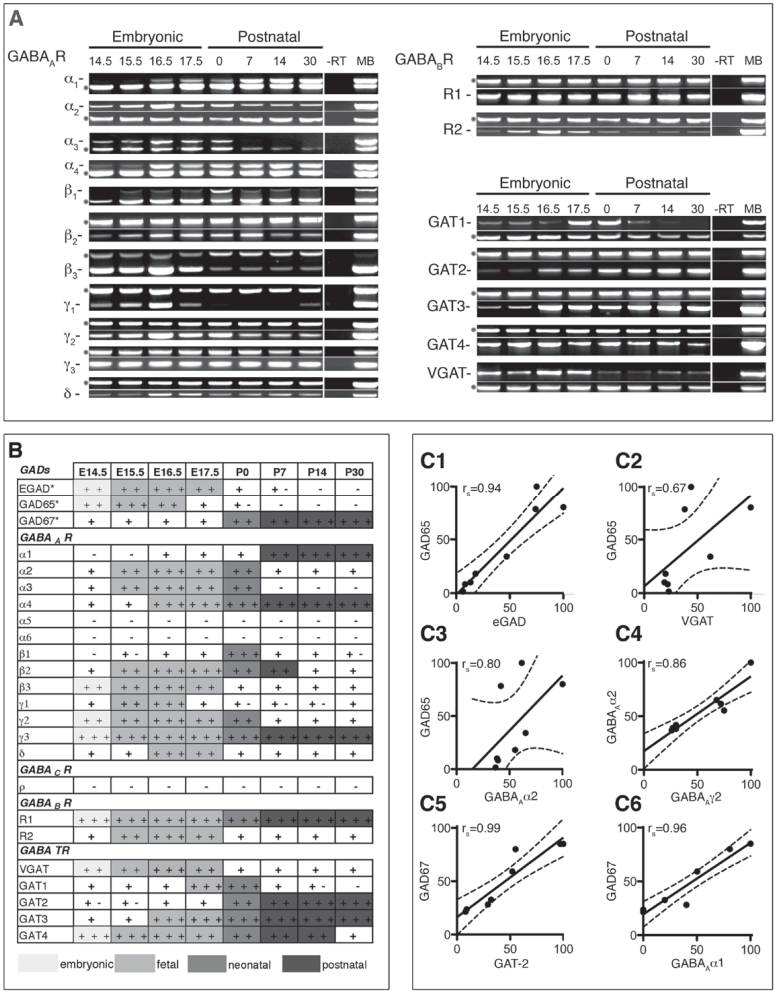

Fig. 17. Temporal expression profile and coordinated expression of genes of GABA signaling pathway in the developing mouse lens. *A*. Expression of $GABA_A$ and $GABA_B$ receptor subunits and GABA transporters as revealed by semi-quantitative RT-PCR. Representative gel images showing the PCR products amplified from reverse-transcribed total RNA that was isolated from embryonic (E14.5-17.5) and postnatal (0, 7, 14 and 30 days) lenses and adult brain (MB). β-actin (*) was co-amplified with the target genes. *B*. Schematic presentation of temporal expression profiles of genes of the GABA signaling pathway in the mouse lens based on the RT-PCR data shown in Fig.1 and Fig.1A in ref. (19). Relative expression level of each gene is indicated as follows: +++, high; ++, medium; +, low; -, no expression. Developmental stages, embryonic: ≤ E14.5, fetal: E15.5-E17.5, neonatal: P0, postnatal P7-P30, were marked by gray scale. *C*. Coordinated expression of GAD, GABA receptor subunits and transporters: eGAD vs. GAD65 (*C1*), VGAT vs. GAD65 (*C2*), GABAAα2 vs. GAD65 (*C3*), GABAAγ2 vs. GABAAα2 (*C4*), GAT-2 vs. GAD67 (*C5*) and GABAAα1 vs. GAD67 (*C6*). Pair-wise correlation analysis of relative gene expression levels at different developmental stages was performed using Prism software. Spearman's rank correlation coefficient (*rs*) for each gene pair is indicated on the scatter plots. The best fit (*solid line*) is shown with 95% confidence intervals (*dotted lines*). Numbers on x- and y-axes represent % of relative gene expression levels.

striking profile being much more abundant before birth, with a transient peak at E17.5, but was hardly detectable after birth. These patterns can be clearly grouped according to different stages of lens development and fiber cell differentiation (Fig. 17B). During embryonic stages mostly GAD65 and the embryonic GAD forms (EGAD) are expressed and this was paralleled by predominant expression of VGAT and the membrane GAT4. The prevalent configuration of the $GABA_AR$ at this stage is $\alpha_{2,3,4}/\beta_3/\gamma_{2,3}$. Most of the GABA signaling components are greatly up-regulated in the fetal lens, in correlation with the high speed of lens growth and generation of lens fibers during this period (Kaufman, 1992). GAD65 and EGAD continued to predominate, paralleled at the beginning by VGAT and GAT4, and later by GAT3 and GAT1 (Fig. 17B). The predicted $GABA_AR$ pentamer during this period is $\alpha_{2-4}/\beta_{2/3}/\gamma_{1-3}$ with all these subunits highly expressed.

P0 was singled out not only because around this stage $GABA_AR$ β_1 and GAT1 were selectively and transiently upregulated, but it also coincided with the suppression of embryonic/fetal components (Fig. 17B).

The postnatal stage is clearly characterized by fewer and highly expressed components. Remarkably, these stages are predominated solely by GAD67, while GAD65 is last detected at extremely low levels at P14. The GAD65-GAD67 switch is paralleled by an almost complete down-regulation of VGAT, $GABA_AR$ α_2, α_3, β_1, β_3, γ_2, δ, $GABA_BR2$ subunits, up-regulation of GAT1,2,3, and the predominantly synaptic in brain $GABA_AR$ α_1 subunit. The Spearman correlation curves clearly showed that there is a high expression correlation between GAD65 and EGAD (r=0.94), GAD65-$GABA_AR\alpha_2$ (0.80), $GABA_AR\alpha_2$-$GABA_AR\gamma_2$ (r=0.86) and somewhat smaller for GAD65-VGAT (r=0.67), which are predominantly expressed before birth (Fig. 17C1-C4). GAD67 expression was highly correlated with that of GAT2 (r=0.99) and $GABA_AR$ α_1 (r=0.96; Fig. C5-6), all expressed postnatally.

5. 2. GABA receptors and transporters exhibit spatially restricted expression in the developing lens

As we demonstrated above at transcript level GABA signaling components are present during mouse lens development. The cellular distribution of VGAT, GAT and the two main GABAR subtypes were studied with specific antibodies (Fig. 18, 19). At E11.5 VGAT, GAT1, GAT3, $GABA_A\beta3$ and $GABA_BR$ showed strong labeling in the lens vesicle, comprised at this stage of a single epithelial layer (Lovicu and McAvoy, 2005). Except for GAT3, which displayed a patchy appearance over the lateral and apical membranes, the staining of all other proteins was identical and greatly enriched at the apical/basal tips of the lens epithelial cell monolayer, especially strong at the apical (luminal) side (Fig. 18A, D, G, J,

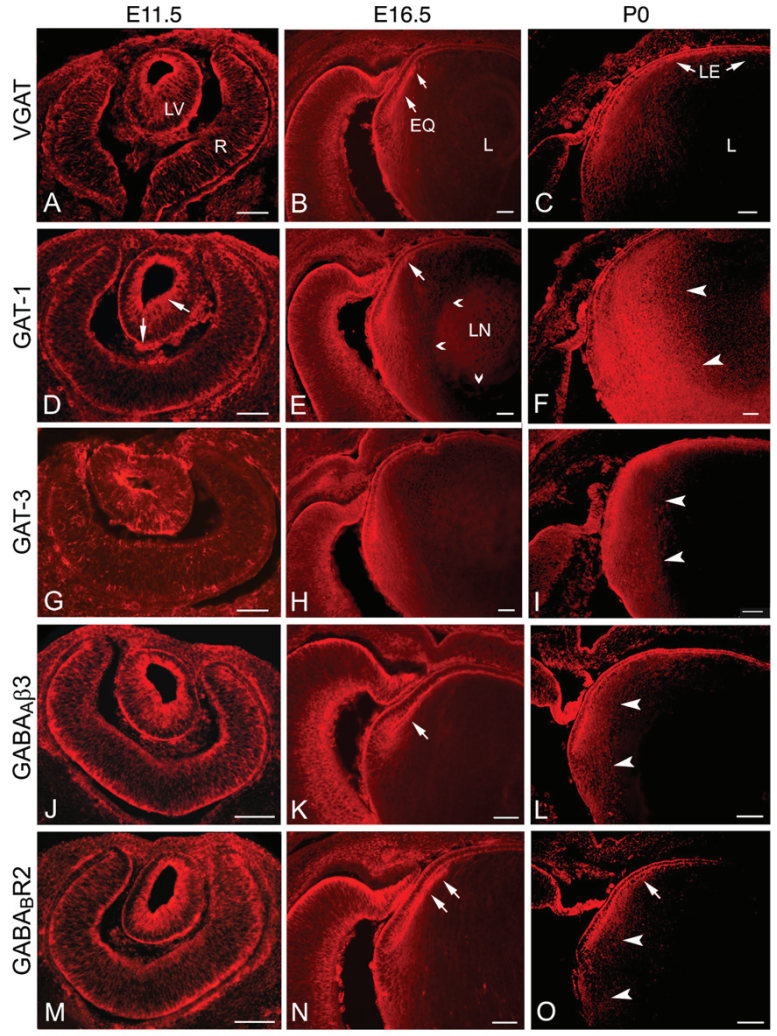

Fig. 18. Cellular localization of GABA transporters and receptors in the developing mouse lens. Coronal cryosections of E11.5, E16.5 and P0 mouse lenses stained with antibodies specific for VGAT, GAT1, GAT3, $GABA_A\beta3$ and $GABA_BR2$. At E11.5 labeling is localized in the lens epithelial cells comprising the lens vesicle (LV) and is greatly enriched at their luminal (apical) and to a lesser extent at the basal ends (A, D, G, J, M; arrows in D). GAT3 is also localized at the lateral membranes conferring a patchy appearance of the LV (G). Both epithelial cells and the tips of secondary fiber cells of the equatorial region exhibit strong labeling for GABAR and GABA transporters at E16.5 (B, E, H, K, N, arrows). Primary fibers forming the lens nucleus (LN) were stained for both GAT1 and GAT3 (E, H, empty arrowheads in E). Compared to E16.5, at P0 GAT1 showed increased expression in the secondary fibers, but was undetectable in the lens nucleus, composed of primary lens fibers (F vs E). GAT1 also exhibited more expanded expression in the cortical region containing secondary fibers compared to VGAT, GAT3, $GABA_AR$ and $GABA_BR2$ (arrowheads in C, F, I, L, O). VGAT and GAT3 expression was decreased and was absent from the lens nucleus compared to E16.5 (B, H vs C, I). Abbreviations: EQ-equatorial region of the lens; L-lens; LE-lens epithelial cells; LN-lens nucleus. LV-lens vesicle; R-retina. Scale bars: 50 μm (A-O).

M). At E16.5, the lens is already polarized with the lens epithelium and anterior suture at the anterior pole, and the posterior suture formed by fusion of the posterior fiber tips at the posterior pole (Kaufman, 1992; Lang, 1999; Ogino and Yasuda, 2000; Zampighi et al., 2000). The lens nucleus (Fig.18E) is comprised exclusively of primary lens fiber cells at various stages of final differentiation and is covered superficially by elongating secondary fibers. At this stage staining with all antibodies was weak in the lens nucleus and much stronger in the equatorial secondary fibers and overlaying lens epithelium (Fig. 18B, E, H, K, N). $GABA_AR$, $GABA_BR$ and VGAT were greatly enriched in the apical/basal membranes of both epithelium and fiber cells showing especially prominent staining in the posterior sutures (Fig. 19A, B, G-J). 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 (Fig. 19C, D), 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 (Fig. 19E, F).

The E16.5 expression pattern was virtually preserved through P0, except for GAT1, which spanned a larger expression domain compared to E16.5 (Fig. 18F), in accordance with the elevated expression of the transcript at this stage (shown in Fig. 17). Both GAT1 and GAT3 were abundantly expressed at the lateral fiber membranes (Fig. 18F,I). In comparison, VGAT was highly enriched at the apical/basal membranes but was never detected on the lateral surfaces (compare Fig. 18C to F, I) and this pattern of staining was also followed by the two GABA receptors (Fig. 18L, O).

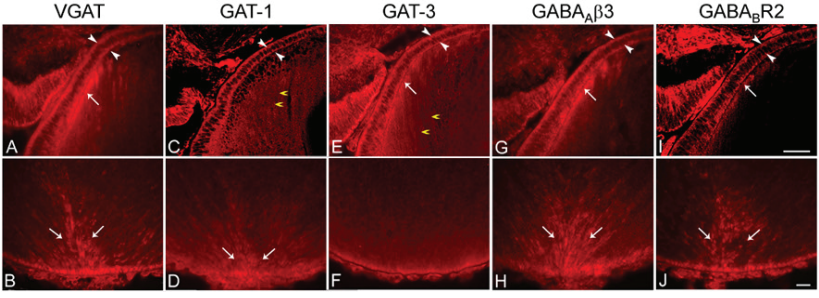

Fig. 19. Subcellular localization of the GABA receptors and transporters in the E16.5 lens: high magnification images of coronal cryosections of E16.5 embryos representing part of the equatorial lens region containing elongating secondary fiber cells (A, C, E, G, I) and posterior lens pole with the forming suture (B, D, F, H, J). Immunoreactivity for $GABA_A\beta_3$ and $GABA_BR2$ receptor subunits and VGAT is predominantly localized to the apical/basal membranes of lens epithelial cells (white arrowheads in A, G and I), apical ends of equatorial fibers (arrows in A, G and I) and their basal tips forming the posterior lens suture (arrows in B, H and J). GAT1 and GAT3 are also seen in the lateral secondary fiber membranes (yellow arrowheads in C, E). Note that GAT1 staining shows less polarized pattern compared to VGAT in the epithelium and apical fiber tips (C vs A), but is enriched at the posterior suture- at the ends of elongating fibers (arrows in D). GAT3 shows a polarized apical/basal expression in the epithelium (arrowheads in E) and enrichment at the apical tips of equatorial fibers (arrow in E), but is not detectable at the posterior suture (F). C and I are confocal images. Scale bars: 50 µm (A, C, E, G, I), 20 µm (B, D, F, H, J).

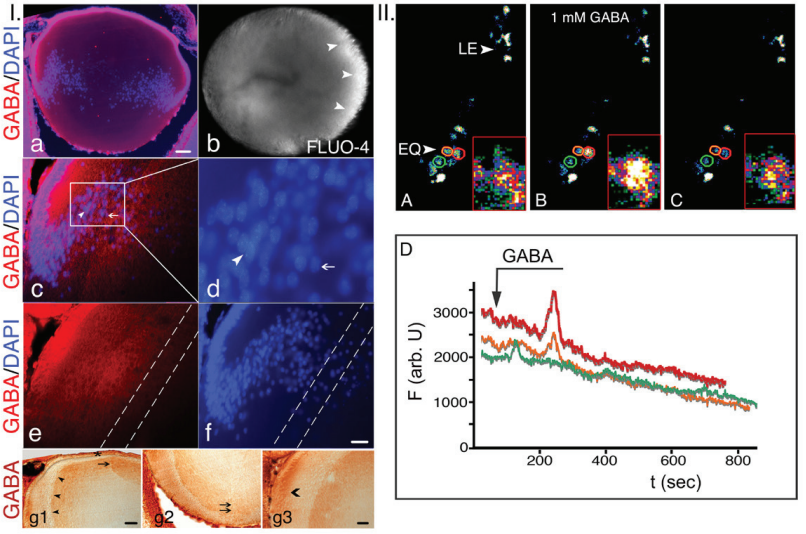

Fig. 20. GABA expression and function in modulation of $[Ca^{2+}]_i$ in the neonatal lens. I: Staining with GABA antibody of the neonatal mouse lens (a, c, e- Cy3; g1-g3-DAB). Nuclei were visualized by DAPI staining (a, c, d, f). GABA immunoreactivity is visible predominantly in equatorial secondary fibers possessing intact nuclei (arrowhead in c, d) and in primary fibers with nuclei in the process of disintegration (arrow in c, d). The area between the dashed lines in e and f includes fiber cells undergoing denucleation and decrease of GABA staining. The border between primary and secondary fibers (arrowheads in g1) and the lens sutures (arrows in g1-g2) are well demarcated by strong GABA staining as is the equatorial lens region containing the newly generated secondary fibers (hollow arrowhead in g3). A P0 lens loaded with Fluo-4/AM (b). **II:** Images from a representative recording of the fluorescence emission in three cells (shown in D in three different colors) of the equatorial region of a Fluo-4/AM loaded newborn mouse lens in buffer alone (A), at the addition of 1 mM GABA (B) and after GABA washout (C). The cells responding to GABA are circled in A, B and C in colors matching the respective recordings shown in D. arb.U-arbitary units; EQ-equator; F-fluorescence; LE-lens epithelium; t-time. Scale bars: 100 µm in a-b; 50 µm in c-f, g1; 20 µm in g2, g3.

6. GABA signaling is functional in the developing lens: activation of $GABA_A$ and $GABA_B$ receptors leads to increase of intracellular Ca^{2+} level in lens cells

6. 1. GABA is produced in epithelial and fiber cells and induces rise of $[Ca^{2+}]_i$ in equatorial cells of intact newborn lenses

In the P0 mouse lens GABA was predominantly localized in the lens epithelium and fiber cells with the strongest labeling found at the equatorial region and the forming sutures (Fig. 20. I. a, c, g1-g3), a pattern entirely consistent with the reported expression of the two GAD forms. An inward gradient of GABA staining in the lens cortex and nucleus follows the progressive fiber maturation and denucleation: the signal declined towards the border of secondary/primary fibers as secondary fibers loose their nuclei (Fig. 20. I. c, g1) but became strong again in external, still nucleated primary fibers (Fig. 20. I. c-f; g1-g3). The nuclei followed a concomitant change in shape and chromatin compaction followed by nuclear

membrane disintegration (Fig. 20. I. c-f). Our results show that GAD and GABA synthesis is turned off shortly before primary/secondary fiber denucleation.

To test the functionality of the GABA signaling in intact lenses we performed Ca^{2+} imaging using laser-scanning microscopy and a specially designed perfusion system (Fig. 20. II. A-D). Lenses were loaded with 5 μM fluo-4/AM, supplemented with 20 % pluronic acid (Fig. 20. I. b) to increase the loading of the dye. In the intact lens only epithelial and equatorial fiber cells were loaded with fluo-4, since they have sufficient esterase activity to load the ester forms of dye (Bassnett et al., 1994).

GABA evoked an increase in fluorescence intensity mostly in cells of the equatorial region (Fig. 20. II. A-EQ) that are undergoing transition from epithelial to fiber cells. Usually, several equatorial cells reacted simultaneously with a moderate increase in fluorescence amplitude. Application of KCl (at 20 mM) after GABA washout always induced fluorescence increase, even when the response to GABA was low (data not shown) demonstrating the viability of the responding cells. However, a drawback of this method was that only a small fraction of GABA positive cells in the P0 lens could be labeled with Ca^{2+} sensing dyes and borders of the reacting cells were poorly discernible. This could be overcome by performing Ca^{2+} imaging in primary LEC culture, an *in vitro* system successfully used before for similar studies (TeBroek et al., 1994; Churchill et al., 2001).

6. 2. Characterization of LEC cultures

Primary LEC cultures were derived from newborn mouse lenses as they express high levels of GABA signaling components as well as provide an ample supply of easy-to-dissect epithelial and fiber cells. After two weeks primary LEC cultures were comprised of single epithelial cells, epithelial monolayers and randomly distributed lentoids-multilayered cell aggregates that appear to bulge from the surrounding epithelial cells. On the basis of morphological criteria and expression profiling lentoids are considered an equivalent of elongating fiber cells (Menko et al., 1984; TenBroek et al., 1994; Ibaraki et al., 1995; Frenzel et al., 1996; Wagner and Takemoto, 2001). The molecular chaperone αA-crystalline, one of the most abundant proteins of the lens epithelium and fibers, was detected in single epithelial cells, monolayers and multilayered lentoids (Fig. 21A, B), while the small αB-crystallin more abundant in fiber cells localized to lentoids (Robinson and Overbeek, 1996) (Fig. 21D, G). Lentoids, but not single epithelial cells stained strongly for N-cadherin (Fig. 21C), typically expressed in elongating lens fiber cells (Xu et al., 2002), while E-cadherin only stained the membranes of epithelial monolayers, but not lentoids (Fig. 21E, G).

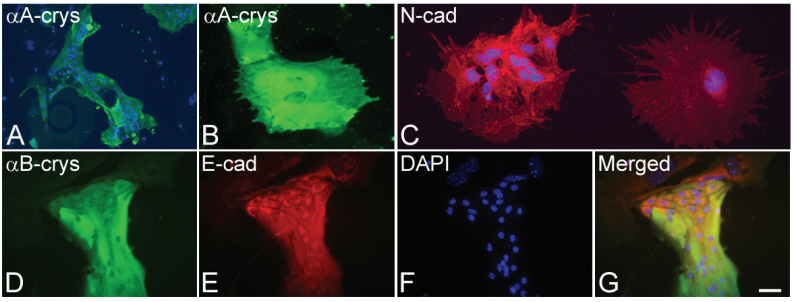

Fig. 21. Characterization of mouse lens epithelial primary cultures. Immunofluorescent staining of primary LECs with antibodies to lens specific αA- and αB-crystallin, E- and N-cadherin. *A, B, D.* As the cells began to differentiate and form lentoids and as the lentoids began to become more highly differentiated, express not only αA- but also αB -crystallin. *C-G.* The polyclonal N-cadherin-specific antibody stains specifically the long (lateral) sides of fibers in lentoids, while epithelial cells and monolayers are not stained. E-cadherin stained only the membranes of epithelial monolayers, but not lentoids. Scale bars: 50 μm in A-C, 20 μm in D-G.

6. 3. Expression of GABA signaling components in LEC cultures

Similar to intact lenses primary LEC cultures prepared from P0 lenses expressed GAD65 and GAD67 forms that co-localized with the vesicular and membrane GABA transporters, respectively (Fig. 22A-L). In general, lentoids showed the strongest expression, while epithelial monolayers were less prominently stained (Fig. 22E, H).

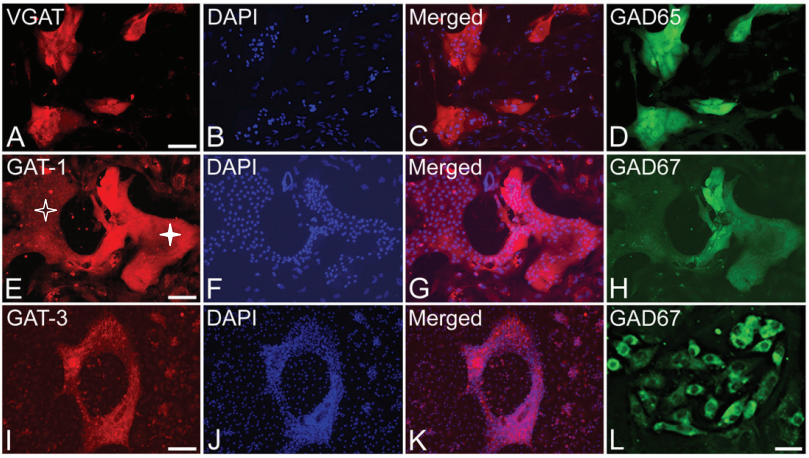

Fig. 22. Expression of GAD, GABA transporters and receptors in primary LEC cultures. Primary LEC cultures derived from P0 mouse lenses showed strong staining for different components of the GABA signaling pathway predominantly in multicellular lentoids, but also in single cells. *A-D.* VGAT entirely co-localizes with GAD65; staining of the same preparation with the polyclonal anti-VGAT serum and monoclonal anti-GAD65 antibody GAD-6 (A, D), counterstained with DAPI (B, C). *E-H.* Co-localization of GAT1 and GAD67: double staining with anti-GAT1 and anti-GAD67 antibody (E, H), staining of the nuclei is with DAPI (F, G). The membrane localization of GAT1 is more evident in the less prominently stained epithelial monolayer (empty asterisk) than the strongly positive lentoid (solid asterisk) (H). *I-K.* Staining with anti-GAT3 antibody reveals predominantly membrane localization in lentoid structures. *L.* GAD67 is also found in small cells outside lentoids and monolayers that are positive for αA-crystallin (not shown). Scale bars: 50 μm in A-L.

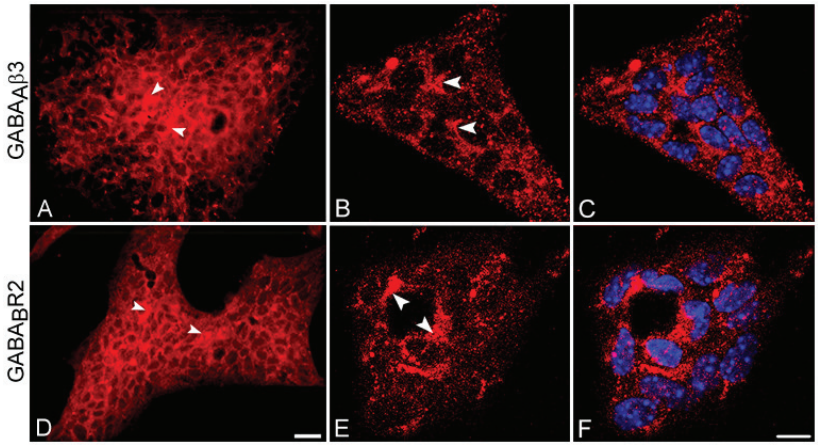

Fig. 23. $GABA_A$ and $GABA_B$ receptors are enriched in multicellular lentoids. LEC cultures were stained with rabbit anti-$GABA_A\beta3$ (A-C) or anti-$GABA_BR2$ (D-F) antibodies and visualized by epifluorescence (A, D) or laser scanning microscopy (B, C, E, F). Nuclei were revealed by DAPI staining (shown in blue color in C, F). The staining has a patchy vesicular appearance and is greatly enriched near cell borders (arrowheads B, E) and at the surface of lentoids (arrowheads in A, D). Scale bars: 20 µm in A, D; 10 µm in B-F.

Strong labeling of the major GABA binding subunit β3 of the $GABA_A$ receptor and the R2 subunit of $GABA_B$ receptor was detected over the cell borders of the lens epithelial monolayers and lentoids (Fig. 23A-F). The staining had a punctate appearance and was greatly enriched near cellular membranes (Fig. 23B, E). Single cells showed little or no staining (data not shown). In conclusion, epithelial sheets and lentoids expressed all GABA signaling components required for GABA action.

6. 4. Primary LEC cultures produces GABA

In parallel with GAD, GABA was also detected in primary LEC. Lentoid structures showed much higher accumulation of GABA compared to adjacent LEC monolayers (Fig. 24a, a'), while single cells remained unstained (Fig. 24c, c'- enhanced for visibility). This expression gradient was typically found in the vicinity of all lentoids and was accompanied by a change in nuclear shape and chromatin condensation reminiscent of the progressive nuclear loss in maturing fibers of intact lenses (Fig. 24b, b'; see also Fig. 20). Thus, primary LEC culture represents a faithful *in vitro* model system of lens epithelial-fiber differentiation that could be used for studying GABA signaling mechanisms.

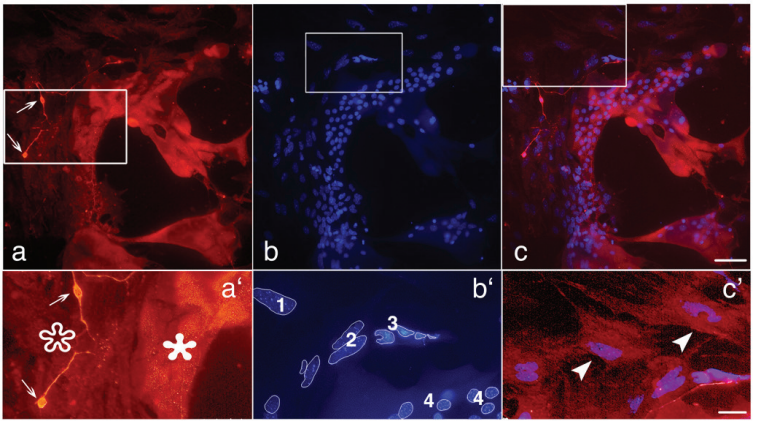

Fig. 24. GABA is produced in LEC cultures. Primary LEC cultures derived from P0 mouse lenses were stained with a rabbit antibody against GABA (a, a’; c, c’). Nuclei were stained with DAPI (b, b’). Boxed regions in a, b, and c are enlarged in a’, b’ and c’, respectively. A large multicellular lentoid-like structure (a-c) is highly positive for GABA in bulging parts (solid asterisk in a’) and less pronouncedly stained at the flatter, presumably monolayer regions (empty asterisk in a’), while cells outside the multicellular structure were unstained (boxed area in c devoid of staining was enhanced in c’ to reveal the presence of cells in this area). Rare $GABA^+$ neurons can be seen (arrow in a) in the preparations that display similar intensity of staining as lentoids (solid asterisk in a’). *b’*-circled nuclei of different shapes and level of chromatin compaction (numbered 1-4) correlating with stages of fiber cell differentiation proceeding from less differentiated (nuclei #1, 2) to more differentiated (nuclei#3 and 4) stages (91). Scale bars: 50 µm in a-c, 20 µm in a‘-c‘

6. 5. *GABAR-mediated modulation of $[Ca^{2+}]_i$ levels in primary LEC cultures*

The functional assembly and activity of $GABA_A$ and $GABA_B$ receptors expressed by primary LEC was verified by measuring $[Ca^{2+}]_i$ changes in cultures upon bath application of GABA and selective agonists/antagonists. Most of the medium size lentoids and single cells were successfully loaded, while some larger lentoids, likely to represent a later stage of lens fiber cell differentiation (Churchill and Louis, 2002) remained unlabeled (data not shown).

GABA (1mM), the $GABA_AR$ agonist muscimol (either 30 µM or 100 µM) and $GABA_BR$ agonist baclofen (20 µM), applied in AAH induced a simultaneous increase of fluorescence intensity mostly in cell groups belonging to smaller lentoids (Fig. 25A1, C1), but also in epithelial cells (cell doublets or monolayers; Fig. 25B1). The $GABA_AR$ agonist muscimol-evoked $[Ca^{2+}]_i$ rise was blocked in the presence of the $GABA_AR$ antagonist bicuculline (0.5±0.34, n=6; amplitude 19.52±5.84%) demonstrating the specific involvement of the receptor in the response (Fig. 25B1, B2, D). Lower number of cells responded to GABA (2.45±0.42, n=20) than to muscimol (4.2±1.01, n=10; Fig. 25D1), but the amplitude of their responses was almost identical (48.06±9.26 % and 46.5±5.49 %, respectively; Fig. 25D2). However, this difference may be incorrectly estimated due to difficulties in defining the contours (and ultimately the exact numbers) of reacting cells within the reacting lentoids.

The $GABA_BR$ agonist baclofen induced small Ca^{2+} transients in single lentoid cells (data not shown). The baclofen-evoked transients in single cells had the smallest amplitude, which could be explained by the low number of receptors, their low sensitivity to baclofen or the presence of baclofen-evoked hyperpolarizing responses. The response to baclofen was typically completely blocked in the presence of the $GABA_BR$ antagonist CGP55845. Continuous application of CGP55845 + baclofen sometimes induced a secondary wave of signal propagation spreading further into the lentoid cells and adjacent epithelial monolayer parts and followed by persistent oscillations (Fig. 25C1, C2), which sustained even after complete washout of CGP55845 (Fig. 25C1, C2). This observation was highly reminiscent of responses involving mobilization of intracellular Ca^{2+} in lens cultures mediated by ATP-receptors and strongly suggests that it may be propagated through intercellular gap junctions and/or purinergic receptors (Duncan et al., 1993; Churchill et al., 1996, 2001; Williams et al., 2001).

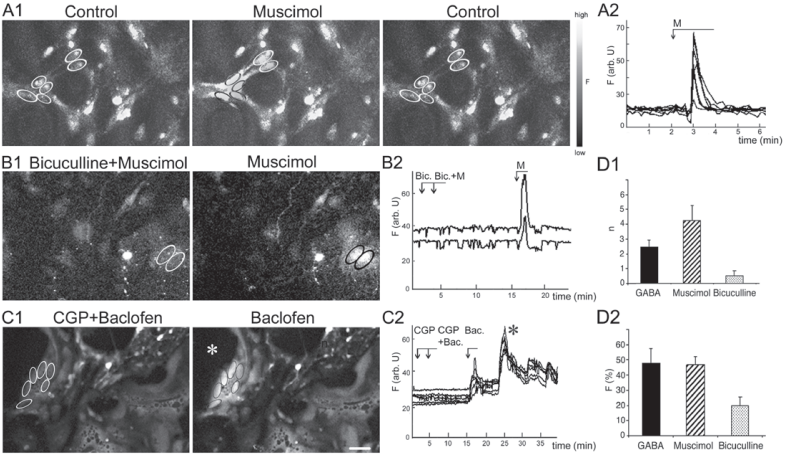

Fig. 25. Activation of GABA receptors exhibits an elevation in intracellular Ca^{2+} in lentoids and epithelial lens cells. *A1, A2, B1, B2.* Representative recordings of $GABA_A$ receptor-evoked changes in fluorescence of Fluo4/AM-loaded primary lens cells (circled). Cultures were initially perfused with AHH buffer to obtain a steady baseline (Control) followed by 100 µM muscimol (Muscimol; A1, A2). Alternatively, 100 µM muscimol and 20 µM bicuculline were co-administered (Bicuculline+Muscimol) followed by 100 µM muscimol (Muscimol) alone (B1, B2). Note that the muscimol-evoked fluorescence rise was completely blocked by bicuculline (B1, B2). *C1, C2.* Baclofen-induced changes in fluorescence in Fluo-4/AM-loaded LEC. Cells were initially washed for 2 min with AHH buffer containing 20 µM CGP55845 (CGP) followed by 20 µM CGP+20 µM baclofen (CGP+Baclofen) and 2 min of baclofen alone (Baclofen). Asterisk in C2 marks the rise of fluorescence in the cells shown in C1-Baclofen labeled by asterisk. Note that baclofen induces a simultaneous rise of fluorescence in cell groups within lentoids (circles in C1) followed by prolonged oscillations (C2). Also, CGP at 20 µM completely blocks the baclofen response (C1, C2). A1, B1, C1 images were taken at different time points of the recordings shown in panels A2, B2, C2, respectively. F (%)- change in fluorescence intensity. *D1-2.* Bar chart illustrating the average number of cells (n in D1) and the amplitude change (F in D2) responding to GABA, muscimol alone and muscimol in the presence of bicuculline. F (%) represents the change in fluorescence intensity relative to the basal fluorescence measured at the beginning of experiment. Data represent the means ± S.D. ($p < 0.05$). Scale bar: 50 µm.

7. Generation and characterization of transgenic mice overexpressing GAD67 in the lens

To investigate possible roles of GABA signaling in lens during development, we generated transgenic mouse models with elevated GABA content specifically in the lens and changes in lens development were examined. To this end we overexpressed the 67 kDa form of glutamic acid decarboxylase in the lens by using the αA-crystallin promoter (Overbeek et al., 1985). This promoter has been shown to be activated as early as GAD and to drive transgene expression in the lens (Reneker et al., 1995).

By using the Crys-GAD67-GFP DNA costruct, we have generated three independent transgenic lines (see detailes in Methods section). Since the phenotypic consequences of transgene expression in the hemizygous state were mild, each of the lines were bred to homozygosity and maintained through interbreeding of homozygous animals. A range of morphological defects was observed in all three lines of CrysGAD67 transgenic mice. The presence of very similar lens specific phenotypes in three lines generated precluded the possibility that changes were due to transgene-mediated insertional mutagenesis. One of the lines (#682) was choosen for further analysis.

7. 1. CrysGAD67 transgene overexpression correlates with elevated GABA levels in the lens

7. 1. 1. Expression of the CrysGAD67 transgene in developing lens

7. 1. 1. 1. SV40 in situ hybridization

Despite the observation that endogenous αA-crystallin is expressed in epithelial cells of the lens (Robinson and Overbeek, 1996) previous analysis has shown that when used in transgenic mice, the αA-crystallin promoter is restricted in its expression to fiber cells (Treton et al., 1991; Robinson et al., 1995). To determine whether the CrysGAD67 transgene showed the same pattern of expression, we performed non-radioactive in situ hybridization on histological sections with digoxigenin labeled transgene specific riboprobe of SV40 origin. Antisense SV40 riboprobe hybridized to non-transgenic eyes and a sense strand riboprobe hybridized to CrysGAD67 lens were used as negative controls. No specific hybridization was seen in any of the negative controls. Based on the results of in situ hybridization, transgene expression was present both in lens epithelial and fiber cells. At E13.5 (Fig.26. I. B) and E15.5 (Fig. 26. I. D), transgene derived mRNA was found in the epithelial and differentiating fiber cells. By P0 transgene expression became stronger and at P0 (Fig. 26. I. F) and P30 (Fig. 26 I. H) lens positive labeling was restricted to epithelial and newly differentiating secondary lens fiber cells at equatorial region, an observation consistent with the expression of the endogenous murine αA-crystallin gene (Treton et al., 1991).

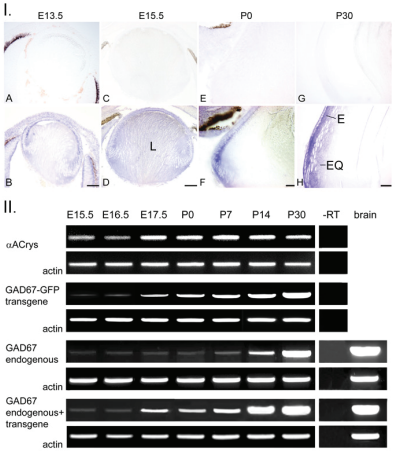

Fig.. 26. Expression of the CrysGAD67 transgene in developing lens. I. In situ hybridization with an sense (A, C, E) and antisense (B, D, F) DIG-labeled riboprobe of SV40 origin. Coronal sections are from E13.5 (A, B), and P0 (C, D), P30 (E, F) postnatal lenses. A sense probe produced no detectable signal (A, C, E). Abbreviations: EQ-equatorial region of the lens, L-lens, E- lens epithelial cells. Scale bar: A-D 100μm; E-H 50 μm; **II.** Developmental expression of endogenous αACrystallin, αACrys-GAD67-GFP transgene, endogenous GAD67 and total (endogenous plus transgenic) GAD67 revealed by RT-PCR. Transgene expression level highly increased from E17.5 to P30 in correlation with elevated endogenous αACrystallin expression. GAD67 mRNA (endogenous plus transgenic) showed elevated expression level in transgenic lenses due to overexpression of transgene-encoded GAD67.

7. 1. 1. 2. Expression of transgene-encoded and endogenous GAD67 transcripts in CrysGAD67 transgenic lenses as revealed by RT-PCR

Total RNA was isolated parallel from the embryonic and postnatal transgenic and wild-type lenses. Expression of the transgene derived CrysGAD67-GFP transcript in the lens was assayed by RT-PCR using a combination of GAD67 and GFP specific primers, which amplifies only the CrysGAD67-GFP hybrid mRNA, but not the endogenous GAD67 mRNA (see Materials and Methods). As predicted a 605 bp fragment was amplified from the transgenic RNAs (Fig. 26. II.). The absence of genomic DNA contamination in isolated lens RNA samples was confirmed by PCR on lens RNA which had not been reverse transcribed into cDNA.

Initial CrysGAD67 expression was detected at E15.5 (Fig. 26 II.), the earliest stage analyzed, in agreement with the appearance αACrystallin promoter driven transgene expression at this stage of lens development (Overbeek, 1985). Transgene expression level highly increased from E17.5 to P30 in correlation with elevated endogenous αACrystallin expression (Fig. 26. II.).

The overall GAD67 mRNA level in the transgenic lenses, including endogenous and transgene encoded, were estimated by GAD67-specific RT-PCR as described in Materials and Methods section (Fig. 26. II.). Using GAD67 specific primers, which recognize both endogenous and the CrysGAD67 transgene derived transcripts, we detected elevated level of GAD67 mRNA (endogenous plus transgenic) in transgenic lenses due to overexpression of transgene-encoded GAD67 (Fig. 26. II.). On the other hand, endogenous GAD67 expression compared to wild type did not change as revealed by RT-PCR using a reverse primer for the

amplification, which was derived from the 3'-untransleted region of the GAD67 transcript not included in the transgene.

7. 1. 2. GAD protein expression in CrysGAD67 transgenic lenses

Immunohistochemical analysis using GAD (serum #6799) antibody was performed to specifically localize GAD in transgenic and wild-type lenses (Fig. 27. I.). Similar to GAD67 expression at RNA level, no major difference in GAD immunohistochemical labeling was observed comparing embryonic and early postnatal transgenic and wild-type lenses (data not shown). However in P30 adult transgenic lens the expression pattern of GADs has significantly changed: GAD immunostaining was extended in the equatorial region, where newly formed secondary fiber cells start to elongate and differentiate (Fig. 27. I.B). The adult P90, transgenic lenses showed even more increased GAD67 expression (Fig. 27. I.D).

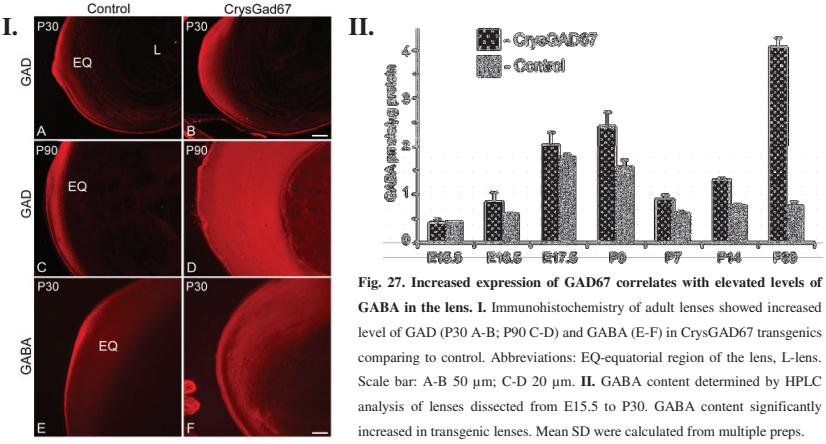

Fig. 27. Increased expression of GAD67 correlates with elevated levels of GABA in the lens. I. Immunohistochemistry of adult lenses showed increased level of GAD (P30 A-B; P90 C-D) and GABA (E-F) in CrysGAD67 transgenics comparing to control. Abbreviations: EQ-equatorial region of the lens, L-lens. Scale bar: A-B 50 µm; C-D 20 µm. **II.** GABA content determined by HPLC analysis of lenses dissected from E15.5 to P30. GABA content significantly increased in transgenic lenses. Mean SD were calculated from multiple preps.

7. 1. 3. Elevated GABA content in the CrysGAD67 lenses

To determine if GABA levels changed with elevated GAD mRNA levels we performed HPLC and immunohistochemistry (Fig. 27. I. E, F; II.). By HPLC we detected elevated level of GABA from embryonic day 16.5. The moderate increase of GABA content in transgenic lenses was proportional at different developmental stages from E16.5 to P14. However, in transgenic P30 samples GABA content increased dramatically by 438% of wild-type level (Fig. 27. II.).

In adult control lenses GABA is found only in the epithelial and newly differentiating fiber cells (Fig. 27. II. E). Whereas GABA showed extended staining pattern in the equatorial region of the adult transgenic lenses similar to that of GAD67 (Fig. 27. II. F). The increased

GAD67 and GABA expression in the equatorial region of the adult transgenic lens was in good agreement with the HPLC results.

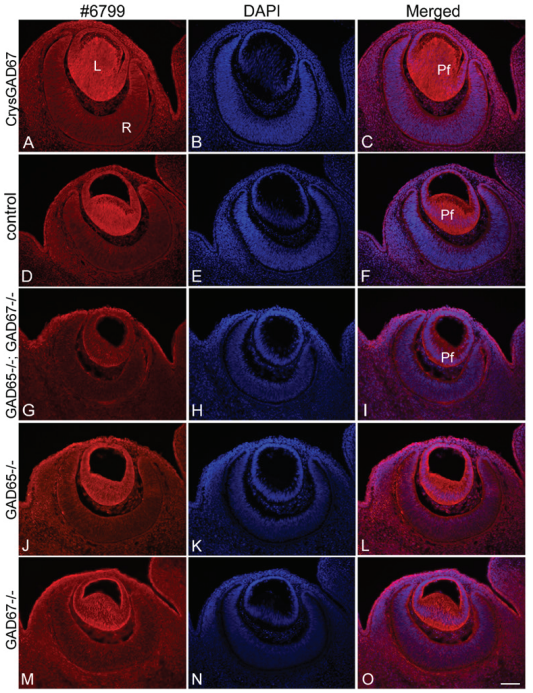

Fig.. 28. Disturbed primary fiber cell elongation in transgenic lenses with genetically altered GAD expression. Coronal sections from E12.5 eyes were stained with anti-GAD serum #6799 and DAPI. In wild-type lens, after closure of the lens vesicle at E11.5 the posterior cells begin to elongate and differentiate into the primary fiber cells at E12.5 (D-F). In the CrysGAD67 mice GAD expression was increased in the lens and primary fiber cell elongation was accelerated (A-C). In the GAD65-/-; GAD67-/- transgenic lenses GAD labeling was dramatically decreased, the lens vesicle stage was prolonged, and the posterior fiber cells failed to elongate (G-I). Lens from GAD65 single knock-out mouse showed similar phenotype (J-L), but in GAD67 single knock-in mice primary fiber cell elongation did not change (M-O), suggesting that GAD65, but not GAD67, activity is required for the initiation of primary fiber cell elongation. Abbreviations: L-lens; Pf-primary fiber cells; R-retina. Scale bar: 100 µm.

8. Genetically altered GAD expression in the mouse lens results in multiple ocular defects

8. 1. Genetically altered GAD expression influences primary fiber cell differentiation

To study the early morphological and molecular changes associated with the overexpression or lack of GADs in the lens, we compared the morphology of wild-type and transgenic lenses starting form embryonic 10.5, just before the disruption in lens development was first evident.

The initial formation of the lens vesicle was not affected in mutant lenses. The first obvious morphological change was detected as early as E12.5. Wild-type lenses showed the typical E12.5 morphology, the posterior lens cells began to elongate and differentiate into the primary fiber cells (Fig. 28D-F). In contrast, the fiber cell elongation program in lenses from homozygous CrysGAD67 transgenic mice (line #682) appeared accelerated. At this stage of development, the transgene has been expressed for a period of approximately 1.5 day. In the

GAD65-/-; GAD67-/- transgenic lenses GAD immunolabeling was dramatically decreased due to inactivation of both GAD genes and the lens vesicle stage was prolonged, primary fiber cell elongation did not initiate until after E12.5 (Fig. 28G-I). The morphology of single GAD65-/- lenses were similar to GAD65-/-; GAD67-/- ones (Fig. 28J-L), however, GAD67-/- fiber cells showed the typical morphology of E12.5 stage (Fig. 28M-O). Beginning at E12.5, in CrysGAD67 transgenic mice a small increase, in GAD65-/-; GAD67-/- double mutant and GAD67-/- single knock-in mice reduction, of the overall size of the eye was also observed. GAD65-/- single knock-out eyes had normal size (Fig. 28J-L). In GAD65-/-; GAD67-/- transgenic lenses at E13.5, incomplete primary fibre elongation is noticeable by the hollowed appearance of the lens, which becomes more obvious by E14.5 (data not shown).

The overall structure of the lens at later embryonic and newborn single GAD65-/- and GAD67-/- transgenic mice did not differ significantly from that of the non-transgenic animals. In addition, the retina in the GAD67-/- mice appeared also normal, but GAD positive cells exhibited atypical distribution (data not shown).

8. 2. Elevated GABA level results in multiple ocular defects

To evaluate the disturbance of eye development induced by overexpression of the GAD67 transgene, ocular histology of late embryonic and postnatal transgenic mice was also analyzed. In CrysGAD67 mice, late embryonic (E17.5) and postnatal eyes were clearly abnormal. Transgenic CrysGAD67 eyes exhibited ocular abnormalities, which fell into two general categories. Some of the morphological changes were restricted to the lens, but others also affected ocular tissues such as the neural retina, cornea and cilliary body.

8. 2. 1. Altered lens fiber structure in transgenic mice overexpressing GAD67

The crystalline lens seems to be simple organ, however it has a highly ordered complex structure, consisting of highly specialized fiber cells.

Gross histological examination of adult CrysGAD67 lenses revealed structural abnormalities, especially in the fiber cell morphology. The fiber cells were excessively disorganized as compared to fibers in wild-type lenses (Fig. 29A-C). In addition, the shape and arrangement of fibers in the transgenic lenses showed complete lack of uniformity (Fig. 29B, C). Abnormalities in the lens fiber cell organization were noticeable in postnatal lenses of CrysGAD67 transgenic mice and became progressively more severe with time (Fig. 28, data not shown).

Defects associated with the posterior sutures were the major morphological abnormalities found in the CrysGAD67 lenses. The fibers failed to form typical Y sutures

(Fig. 29D-F). Posterior suture defects observed by bright-field microscopy in older lenses could be detected as a cleavage in the tissue surface. Large gaps were also observed in the area where the posterior suture lines would normally be located (Fig. 29E, F).

Adult CrysGAD67 lenses were also opaque; the degree of loss of transparency was correlated with the abnormal fiber structure.

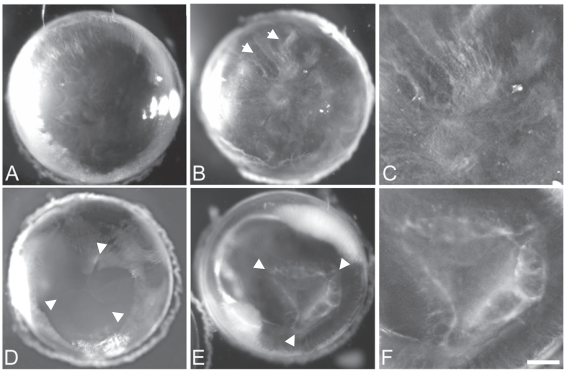

Fig. 28. Stereo photomicrographs of unfixed adult wild-type (A, D) and CrysGAD67 transgenic (B, C, E, F) mouse lenses. A-C: An anterior view of lenses. The shape and arrangement of fibers in the transgenic lenses were less uniform (B, C) compared to the control (A). D-F: A posterior view of mouse lenses. Wild-type lenses formed the typical „Y" suture (D). In contrast, CrysGAD67 transgenic lens fibers faild to contact and form this „Y" suture (E, F). C, F are higher resolution images of B and E panel. Scale bar: 500 μm (A, B, D, E), 200 μm (C, F).

8. 2. 2. Overexpression of GAD67 in the lens causes epithelial hyperplasia

The epithelium at the anterior surface of the lens epithelium of lenses of wild-type mice is normally composed of a single layer of tightly packed cuboidal cells (Fig. 30M). The epithelial monolayer was one of the first affected structure in the lenses of CrysGAD67 transgenic mice. Beginning at P0, signs of epithelial hyperplasia, represented by foci of cellular disorganization and proliferation, were observed in the mutant lenses. The lens epithelium in CrysGAD67 transgenic adult eye was multi-layered (Fig. 30J, N, T), the central epithelial region became progressively more hyperplastic. By comparison, the epithelium of control lenses retained an orderly monolayered arrangement (Fig. 30M).

8. 2. 3. Epithelial cell proliferation is altered by genetic modification of GAD expression in the lens

Studies in the CNS and non-neural tissues have shown that GABA can influence cell proliferation (Minuk et al., 1993; Varju et al., 2000; Tamayama et al., 2005; Owens and Kriegstein, 2002; Watanabe et al., 2006). To determine if genetic aleration of GAD expression in the lens, overexpression of GAD67 or lack of GAD67, influences cellular proliferation, cells that stained with Ki-67 proliferation marker were counted in lenses from wild-type, homozgous CrysGAD67 and homozygous GAD67-GFP knock-in mice.

Compared to control non-transgenic lenses, no obvious change in the number of Ki-67-

positive cells were detected in the lens at early embryonic stages in CrysGAD67 and GAD67-/- mice (data not shown, examined from E10.5). At E16.5 there was still no significant differences between the populations of proliferating cells in CrysGAD67 transgenic (460±15) and wild-type (476±39) lenses (t=0.76; P<0.05; n=4) (Fig. 29). However, at late embryonic and neonatal stages, Ki-67 labeling in lens epithelial cells was significantly increased in the CrysGAD67 transgenic mice when compared to non-transgenic mice. At E17.5, there were significaly more KI-67 positive cells in transgenic lenses (694±96) compared to wild-type (427±10; 62% increase, t=5.53; P<0.05; n=4) (Fig. 29). At postnatal stages (P0-P7) proliferation of epithelial cells was still increased in transgenic lenses. There were still significantly more Ki-67- positive cells in transgenic then it control lenses: at P0 (control 334±52 and transgenic 529±15 positive cells) (58% increase, t=4.17; P<0.05; n=4), at P2 (control 312±33 and transgenic 553±99 cells) (77% increase, t=4.59; P<0.05; n=4) and at P7 control 294±28 and 546±70 cells transgenic (86% increase, t=6.69; P<0.05; n=4). At P14 there was no significant differences between the number of Ki-67 positive proliferating cells in transgenic (91±11) and wild-type (77±5) lenses (t=2.3; P<0.05; n=4).

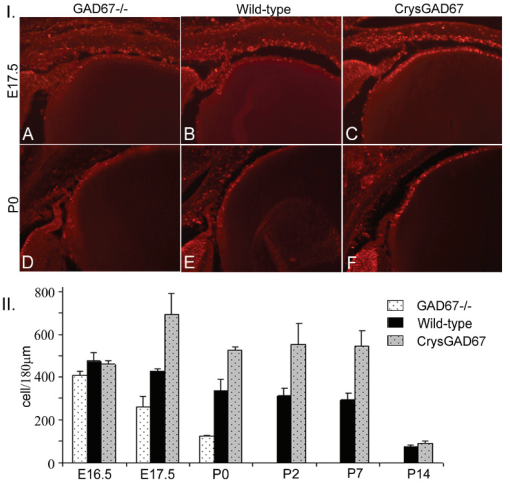

Fig. 29. Quantification of cell proliferation in the lens of CrysGAD67 and GAD67 knock-in mice. I. Immunohistochemistry of Crys-GAD67 and GAD67 knock-in mouse lenses with Ki-67 antybody, which markes actively dividing cells. **II.** Quantification of cell proliferation in the CrysGAD67 and GAD67 knock-in lenses. In Crys-GAD67 mice there is a significant increase in Ki-67 positive cells from E17.5 to P7 (E17.5-62%, t=5.53; P0-58%, t=4.17; P2-77%, t=4.59; P7-86%, t=6.69; P<0.05; n=4). There is no statistical difference in cell proliferation at P14 (18%, t=2.3; P<0.05; n=4). In GAD67 knock-in mice amount of proliferating cells is significantly decreased comparing to the control (E16.5-14%, t=3.32; E17.5-39%, t=6.7; P0-63%, t=6.28, P<0.05; n=4).

As expected, in contrast to CrysGAD67 lenses, the loss of GAD67 expression reduced proliferation of lens epithelial cells. In GAD67-/- mice a slight but significant decrease in Ki-67-positive epithelial cell number was first observed at E16.5 (476±39 in control and 407±20 in KO) (14% decrease, t=3.32; P<0.05; n=4). A marked decrease was observed also at E17.5: 427±10 cells were Ki-67 positive in control and 259±49 in GAD67-/- lenses (39% decrease,

t=6,7; P<0,05; n=4). The difference was more pronounced at P0, 334±52 epithelial cells were positive for Ki-67 in control lenses, 123±6 in GAD67-/- (63% decrease, t=6,28; P<0,05; n=4) (Fig. 29). Since homozygous GAD67-KO mice die at birth, we could not monitor cell proliferation beyond this age.

In the retina and anterior eye structures of CrysGAD67 and GAD67-/- mutant mice, Ki-67 immunohistochemistry indicated similar changes in cell proliferation to that of the lens (data not shown).

8. 2. 4. Anterior ocular tissues are also affected in transgenic mice that overexpress GAD67 in the lens

In transgenic mice that overexpress GAD67 in the lens, there were some developmental disturbances also in ocular tissues anterior to the lens: for instance, thickening of the corneal epithelium and sometimes fusion of iris or cilliary body with the lens (Fig. 30G, O). Corneal adhesion sometimes was seen at the foci of cellular disorganization and hyper-proliferation in the transgenic lens (Fig. 30T). In some cases iris or cilliary body was attached to the lens (Fig. 30G, S), or the lens was fused to the retina (Fig. 30P). These pathological changes represent secondary defects to the lens abnormalities. By comparison, such adhesions were never detected in control eyes (Fig. 30E).

8. 2. 5. Retinal phenotype in transgenic mice that overexpress GAD67 in the lens

Initially we have generated the CrysGAD67 transgenic lines on FVB/N background. Since the standard FVB/N strain homozygous for Pb6b ^{rd1} suffers form retinal degeneration, it cannot be used for evaluation of genes that control retinal development or function (Bowes et al., 1993; Pittler and Baehr, 1991; www. jax.org./jaxmice). For the above reason, in order to study the possible retinal phenotype we had to transfer CrysGAD67 transgenic line to FVB/Ant background, a modified version of FVB/N that lacks Pde6b ^{rd1} (Errijgers et al., 2007).

As expected, sagittal sections through the retina of adult CrysGAD67 mice on FVB/N background revealed the absence of the photoreceptor layer, whereas sections through the retina of same line on FVB/Ant background showed normal, appropriately positioned layer of photoreceptors (Fig. 30F, R, H, P). However, even on FVB/Ant background we detected unusual folding of the retina from E 17.5 (Fig. 30F, R). In postnatal eyes retinal folds became more pronounced and ectopic retina was also developed (Fig. 30D, F, R, H). This finding indicates that the lens phenotype is related rather to overexpression of GAD67 in the lens then to the genetic background.

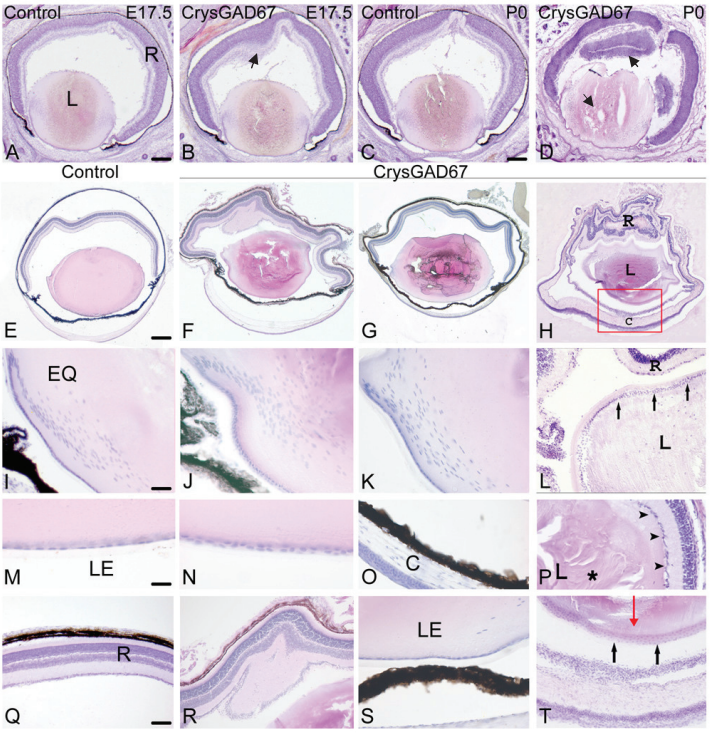

Fig. 30. GAD67 overexpression in the lens of transgenic mice carrying CrysGAD67 transgene causes multiple ocular defects. A-D. Coronal cryosections from eyes of P0 and P2 control (A, C) and transgenic (B, D) mice. Arrows mark folding of the neural retina (B, D) and disrupted lens nucleus (D). E-T. Sagittal sections from contol and transgenic CrysGAD67 mice demonstrates: J, K, L-abnormally positioned cell nuclei at lens equator; N, T-multilayered lens epithelium; F, H, R-retina folding, ectopic retina; P-lens retina fusion; S-lens-cilliary body fusion; O, T-cornea- cilliary body fusion; F-H, P-lens cataract. Abbreviations: C-cornea; L-lens; LE-lens epithelium; R-retina; Scale bar: 100 µm (A-D, Q, R), 500 µm (E-H), 20 µm (I-P, S, T), µm.

8. 2. 6. Altered N-cadherin and Pan-cadehrin expression in GAD67 overexpressing lens correlates with disturbed lens fiber structure

Previous studies have identified a number of proteins in lens fibers that function as cell adhesion molecules or that serve as a links between cytoskeletal elements and components of cell junctions (Takeichi et al., 1986; Franke et al., 1987; Lo, 1988; Lagunowich et al., 1989; Volk et al., 1990). Changes in the expression of these molecules are correlated with events during lens development, like fiber cell elongation, detachment from the lens capsule, association at the sutures. These molecules may be important in stabilizing lens fiber cell plasma membranes or in maintaining cell shape (Beebe et al., 2001).

Since our results indicated that elevated GAD/GABA levels alter fiber cell differentiation and lens fiber structure, we examined the expression of the cell adhesion molecules N-cadherin and Pan-cadherin in the lens of transgenic mice overexpressing GAD67. Figures 31 shows sagittal (A-C, F-H) and coronal (D, E, I, J) sections through lenses at P30, stained with antibodies to N- and Pan-cadherin. In wild-type lenses fibers were of uniform shape and size, and had typical regularly arrayed cadherin expression along their length (Fig. 31A-C, E). Cross sections of lens fibers showed that most of the N-cadherin (data not shown) and pancadherin (Fig. 31E) were detected on the short side of the fiber cells. The lateral membranes of elongating lens fibers were also stained (Fig. 31A-C, E). Immunostaining for N- and Pan-cadherin along the fiber cell membranes decreased in deeper fiber cells soon after they detached from the capsule (Fig. 31B, G).

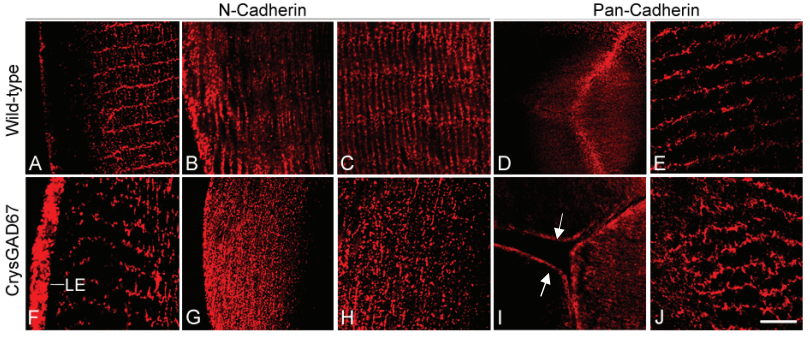

Fig. 31. Immunolocalization of cell adhesion proteins in cryosections of adult wild-type (A-E) and CrysGAD67 (F-J) mouse lenses. Immunofluorescence microscopy of wild-type lenses showing the hexagonal appearance of the fiber cells, with lateral long side and apical short sides, after immunolabeling with antibodies against N-cadherin (A-C, F-H) and Pan-cadherin (D, E, I, J) observed by confocal laser scanning microscopy. In CrysGAD67 lenses distribution of N-and Pan-cadherin expression along the irregularly running fiber cell membranes: increased and abnormal pattern of expression (F-J). In CrysGAD67 transgenic lenses arrows indicate the gap observed in the area of the posterior suture (I). Abbreviations: LE-lens epithelium. Scale bar: 20 µm (A, F, E, J), µm, 50 µm (B, C, D, G, H, I).

Our analysis showed that although CrysGAD67 lenses were grossly of comparable size to wild-type lenses, they had almost no resemblance of normal fiber shape or arrangement. The distribution of N-and Pan-cadherin expression along the fiber cell membranes had disordered expression (Fig. 31F-H, J). The CrysGAD67 transgenic lenses, especially the multilayered lens epithelium showed increased N-cadherin labeling (Fig. 31F). Cross sections stained with Pan-cadherin showed prominent labeling at fiber cell endings. In wild-type lenses as a regular Y shaped staining was detectable along the posterior sutura, where fibers arranged end to end (Fig. 31D). In CrysGAD67 transgenic lenses staining was detectable along the gap observed in the area of the posterior suture (Fig. 31I).

VI. Discussion

To summarize the importance of our findings, we showed for the first time the presence of all necessary molecular components of GABA signaling machinery in the developing mouse lens and provided evidence for their assembly into a functional signaling system by demonstrating that GABA receptor activation induces intracellular calcium rise, which in turn could regulate diverse cellular processes related to lens development and function.

We also demonstrated by genetically altering the expression of GAD, that GABA as a signaling molecule plays an important role in lens development by regulating epithelial cell proliferation, fiber cell elongation/differentiation and fiber cell adhesion.

1. Sequential induction and spatial segregation of different GAD isoforms recapitulate different phases of lens development

In contrast to the cellular and molecular complexities present in most other tissues, the lens is a relatively simple system, composed of a single layer of metabolically active epithelial cells that differentiate into quiescent, but structurally and functionally highly specialized fiber cells (McAvoy et al., 1999). The progress from epithelial to terminally differentiated fiber cells proceeds through well-defined stages characterized by the synthesis of specific sets of intermediate filament proteins (Blankenship et al., 2001), adhesion molecules (Beebe et al., 2001; Zelenka, 2004) and lens crystallins (α, β, γ, δ).

As detailed below, we have demonstrated that the expression of different GAD isoforms highly parallels distinct phases of lens development indicating that GABA synthesized by different GAD forms may have specific roles in different lens compartments where they are expressed during development.

We showed that the four known GAD protein isoforms: GAD65, GAD25, GAD44 and GAD67 are expressed during lens development. The spatio-temporal expression of GAD65 and GAD67-related forms during stages E9.5-P30 (schematically presented in Fig. 32) showed that the GAD65 and GAD67 genes were co-expressed already at the placode stage and their expression essentially overlaps in both lens epithelium and primary lens fiber cells until around P7. During embryonic stages (until E17.5) GAD65 and the embryonic GADs encoded by the GAD67 gene are the predominant forms. On the contrary, only traces of adult GAD67 mRNA, but not the protein were synthesized until late embryonic stages, indicating that it may be present at extremely low levels.

The highest level of expression of both EGAD and GAD65 was observed in the primary

lens fibers at stages E16.5-E17.5 characterized by extensive fiber elongation and beginning of nuclear disintegration. This highly coordinated expression of GAD65 and EGAD strongly suggests they have a synergistic role in the primary fiber differentiation, involving the processes of cell migration, elongation, and withdrawal from the cell cycle.

After E17.5, GAD expression follows the opposite tendency: down- regulation of the ”early” GADs in the lens nucleus and up-regulation of the ”late” GAD67 in the elongating secondary fibers in accordance with the terminal differentiation and enucleation of primary fibers and enhanced secondary fiber generation.

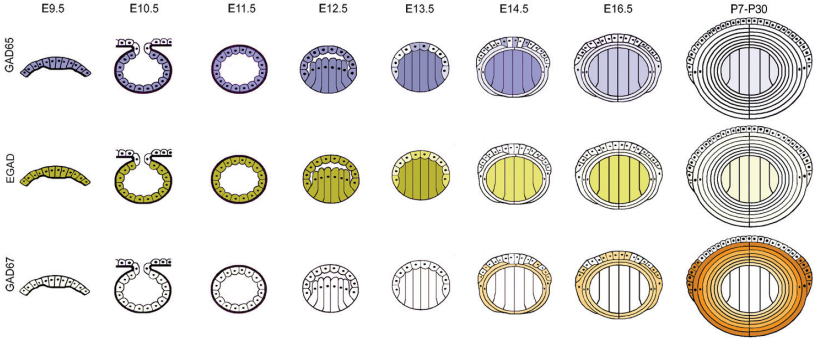

Fig. 32. Schematic presentation of GAD65 and GAD67 expression during lens development. Starting from the lens placode stage to E16.5, GAD65 and GAD67-related (EGAD) expression entirely overlaps (see Discussion). GAD65 expression overlaps with EGAD in the primary fiber cells, where it is down- regulated after P14. At P30 and thereafter, only GAD67 is expressed in the elongating secondary fiber cells and some cells of the epithelium, but not in lens nucleus.

The predominant expression of GAD67 in the nucleated secondary fibers, which undergo much faster transition from elongating GAD positive fibers into the organelle-free (and GAD-free) zone may be explained by the need for a rapid recruitment of constitutively active pyridoxalphosphate (PLP)-bound GAD67. Conversely, GAD65 which is found mostly as an inactive apo-GAD (Martin et al., 2000; Battaglioli et al., 2003; Chen et al., 2003) and enzymatically active GAD44, regulated mostly at the translational level (Szabo et al., 1994) may provide an abundant, but more slowly mobilizable pool of GAD during the extended process of primary fiber differentiation. This notion is supported by the observation that despite the overall decreased GAD synthesis in P0 compared to E17.5 lens, the corresponding GABA level does not drop significantly.

Although the precise role of the embryonic GADs and their possible interaction with the adult GADs are largely unknown even in the nervous system, EGAD-GAD65 co-expression in the lens suggests both synergistic roles in the maintenance the GABA homeostasis and

diverse roles within the cell (Szabo et al., 1994; Varju et al., 2001).

In accordance with their expression pattern, GAD44 along with GAD65 may provide GABA for the early stages of epithelial cell proliferation and primary cell differentiation. Due to preferential accumulation of the "early" GADs at the apical and basal tips of the primary fibers, GABA synthesized by these forms may play a role in remodeling of the membrane skeleton during fiber elongation. Furthermore, the "early" GAD related GABA signaling may be involved in formation of transient cell-adhesion complexes between elongating fiber cells and overlying lens epithelium and the lens capsule at the posterior lens wall. Similarly, the extremely high levels of "early" GADs at the anterior and posterior lens poles and in the central lens epithelium during E13.5-E16.5 suggests that GABA synthesized by GAD44/GAD65 is involved in cell adhesion mechanisms between fibers of opposite sides during formation of lens sutures (Beebe et al., 2001).

The function of the other embryonic GAD form GAD25 is unclear; it is enzymatically inactive as a glutamate decarboxylase, because it lacks the pyridoxal 5'-phosphate-binding site. Similar to the nervous system, GAD25 may regulate the expression of GAD at translational level, and could play a role in establishing GABAergic phenotype during development.

GABA has been also detected at variable levels in the lenses of other vertebrates (Li et al., 1995). The previously reported expression patterns of GAD67 and EGAD in the developing rat lens show remarkable similarities to our results found in the mouse with some notable differences. In E17.5 rat lens (corresponding roughly to E16.5 in the mouse), when the highest levels of GAD are detected, mostly GAD67, but not EGAD is synthesized; furthermore, GAD67 was not detected before E15 and was only expressed shortly after birth. Among these, the absence of GAD67 immunostaining from the postnatal cortical fibers may be due to a much lower level of GAD67 in the rat compared to mouse lens and/or a lower affinity of the GAD67-specific antibody used in this study. This may also help explain the discrepancy between lack of GAD67 staining and the presence of GABA in the postnatal rat lens (Li et al., 1995). Moreover, the expression of GAD65 in the rat lens has not been tested.

The spatio-temporal expression of GAD in the differentiating primary fiber cells of the mouse lens shows a remarkable similarity to the expression of cholineacetyltransferase (Chat, the ACh-synthesizing enzyme) in chicken differentiating lens fibers. Especially noteworthy is the high accumulation of GAD and Chat at the apical and basal tips of the elongating fibers, and the primary fiber cell elongation defects of GAD mutants, also suggesting that GABA may be involved in the fiber elongation process, as suggested for ACh (Oppitz et al., 2003).

A number of nervous system-specific proteins are expressed during defined differentiation stages of the lens (Baechner et al., 1995) indicating the involvement of common mechanisms with the developing nervous system. Striking functional and structural similarities between lens fiber cells and neurons are observed in the regulation of vesicle trafficking and the involvement of Alzheimer (AD) proteins in this process in the normal state and during disease (Frederikse and Zigler, 1998; Li et al., 2003; Frederikse et al., 2004). Similarities between the lens cells and the neurons suggests, that studying GABA signaling in relatively simple system like lens might help us to understand its role in the much more complex nervous system. In addition, the lens affords a unique model system to study the regulation of expression and intracellular role of individual GAD isoforms which, similarly to the developing nervous system (Szabo et al., 1994; Katarova et al., 2000; Varju et al., 2001) are sequentially induced, but are spatiotemporally segregated in the lens.

2. Dlx2 and Dlx5 could serve as potential transcriptional regulators of GADs in the developing lens

Dlx2 and Dlx5 homeobox transcription factors show overlapping expression patterns with GAD65 and GAD67 in the basal forebrain, where they are considered as key regulators of differentiation and migration of the GABAergic neuronal progenitors (Anderson et al., 1997a, 1997b, 1999, 2002; Katarova et al., 2000; Pleasure et al., 2000; Marin and Rubenstein, 2001; Panganiban and Rubenstein, 2002; Cobos et al., 2005). Dlx1&2 and Dlx5&6 gene pairs are expressed in a sequential and overlapping fashion in cell populations residing in the ventricular, subventricular and mantle zones of the developing basal forebrain in the order Dlx2, Dlx1 and Dlx5 and Dlx6, respectively. Detailed studies on the available Dlx knock-out mice have shown that the four genes have partially redundant functions in the cells where they are co-expressed (Liu et al., 1997; Eisenstat et al., 1999; Zerucha and Ekker, 2000; Panganiban and Rubenstein, 2002; Depew et al., 2005). Recent experimental evidence showed that Dlx2 and Dlx5 activate both GAD65 and GAD67 genes in the forebrain (Anderson et al., 1999; Stuhmer et al., 2002a, 2002b). The Dlx family may also regulate GAD in other brain regions including the olfactory bulb and neuronal cell types, such as migratory LHRH neurons as well as in structures outside the nervous system like teeth, vibrissae, palate and the where they show overlapping expression patterns with GAD (Porteus et al., 1994; Thomas et al., 1997; Katarova et al., 2000; Maddox and Condie, 2001; Levi et al., 2003; Depew et al., 2005; Givens et al., 2005; Tamayama et al., 2005; Long et al., 2007).

We found by semi-quantitative RT-PCR that the two Dlx genes are clearly expressed in a sequential manner in the developing mouse lens. Dlx2 expression was induced before Dlx5. Dlx2 shows a temporal profile almost indistinguishable from that of EGAD and very similar to GAD65, suggesting that Dlx2 may be needed for induction and/or maintenance of the "early" GAD forms. In comparison, Dlx5 expression correlates only partially with that of GAD67: its initial up-regulation precedes that of GAD67 (E17.5 vs P0), but unlike GAD67, which shows increasing expression levels at P0-P30, Dlx5 is not expressed beyond P14. These findings strongly suggest that Dlx5 may be needed for induction, but not for maintenance of GAD67 expression. The sequential and overlapping expression of Dlx2 and Dlx5 in the lens is reminiscent of that in the brain (Liu et al., 1997; Eisenstat et al., 1999; Panganiban and Rubenstein, 2002) where it may be related to the sequential induction of EGAD and GAD67 in the differentiating GABAergic progenitors.

Interestingly, we found only subtle differences in the expression of embryonic GADs and GAD67 in E18.5 lenses of Dlx1/Dlx2 double mutants, which would indicate that Dlx2 might be dispensable for GAD regulation during embryonic lens development. Its absence could be be compensated for by other transcription factors of the Dlx family such as Dlx3, which is abundantly expressed in the lens throughout stages E15-P30 (unpublished results; The Gene Expression Nervous System Atlas (GENSAT): http://www.ncbi.nlm.nih.gov/projects/gensat) or Prox1. Dlx3 has been shown to be a key regulator of development of the ectodermal placodes, but its possible role in eye development or regulation of GADs remains unexplored because of the severe phenotype and early embryonic lethality of the knock-out mice (Morasso et al., 1999). A possible candidate transcription factor compensating for the absence of Dlx1/Dlx2 is Prox1, which showed unchanged expression in the E18.5 Dlx1/Dlx2 mutant lens (data not shown, see Kwakowsky et al., 2007). Prox1 mutant lens fiber cells fail to elongate properly resulting in a hollow lens (Wigle et al., 1999). However, the severe embryonic lethal phenotype of Prox1 mutant mice precludes a detailed study on the expression of the GAD genes throughout lens development in this mouse model.

No obvious lens phenotype has been described for Dlx5/Dlx6 knock-out mutants, all showing embryonic or neonatal lethality (Zerucha et al., 2000; Panganiban and Rubenstein, 2002; Robledo et al., 2002; de Melo et al., 2005). This may be explained by the existence of multiple compensatory mechanisms afforded by genetic (expression of multiple genes of the same family) and functional (cross-regulation from different transcription factors) redundancy

of the system during embryonic development, which can result in a later (postnatal) manifestation of the mutant phenotype.

3. All GABA signaling components are expressed and temporally regulated in the developing lens - evidence for co-regulation

The GABA signaling machinery encompasses molecular components involved in GABA synthesis (glutamic acid decarboxylase-GAD), release (vesicular GABA transporter-VGAT and membrane transporter GAT), binding ($GABA_A$ and $GABA_B$ receptors), uptake (GAT) and degradation (GABA-T).

We reported for first time, in addition to the GABA synthesizing enzymes, the presence and the spatial and temporal expression of different $GABA_A$ and $GABA_B$ receptor subunits, vesicular and membrane GABA transporters (vesicular VGAT and membrane GAT) in the developing mouse lens and in primary lens epithelial cultures. As discussed later, we also showed that these components form a fully functional signaling system, since GABA receptor activation evoked transient increase of $[Ca^{2+}]_i$ in intact lenses and in primary LEC.

We showed, 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 RT-PCR data indicate a clear switch from embryonic to postnatal expression of different components. Thus, the subunit composition of $GABA_AR$ changes from the predominant embryonic $\alpha_{2,3}/\beta_3/\gamma_{1,2,3}/\delta$ to the predominant postnatal $\alpha_{1,4}/\beta_2/\gamma_3$ that could result in changes in the receptor kinetics. For instance, an ontogenetic replacement of α_2 with α_1 has been shown to result in a change from a slow decay to fast decay receptor kinetics (Okada et al., 2000). Similarly, a reduction of $\alpha_{2,3}$ subunits with concomitant retainment of α_1-containing, faster decay kinetics receptors in $GABA_AR\beta_3^{-/-}$ mutant mice (Ramadan et al., 2003) or the compensatory up-regulation of α_2/α_3 (and down-regulation of $\beta_{2/3}$ and γ_2) subunits in $\alpha_1^{-/-}$ mutants (Kralic et al., 2002) implies that expression of $\alpha_{2,3}$-β_3-γ_2 is coordinately regulated during development. The presence and selective up-regulation of δ, a subunit, which confers high affinity to GABA, slow rate of de-sensitation and sensitivity to neuroactive steroids (Mihalek et al., 1999; Peng et al., 2004) at late gestational stages may indicate a specific function in the maturation of primary fibers that may be modulated by endogenous steroids. Similar role can be attributed to $\alpha_{2,3,4}$, β_3 and γ_1, which can be differentially regulated by gonadal steroids (Pierson et al., 2005). Although $GABA_AR$ α_5 gene is found clustered with γ_3 and β_3 in the same locus of mouse Chr 7, it does not seem to

interact with γ3 in brain (Togel et al., 1994). The exceptionally high level of γ_3 expression and the total absence of the closely positioned α_5 (Fig. 17) indicate that they are oppositely regulated at the transcriptional level throughout lens development. A coordinated transcriptional regulation has recently been demonstrated for the $\beta_2-\alpha_6-\alpha_1-\gamma_2$ subunit gene locus as well (Uusi-Oukari et al., 2000).

In brain and peripheral tissues $GABA_BR1$ subunit is much more abundant than $GABA_BR2$, which is critical for trafficking the receptor to the membrane (Thuault et al., 2004). Similar correlation was observed for $GABA_BR$ of the prenatal lens. However, the extremely low level of $GABA_BR2$ in the postnatal lens may present a limitation for the formation of functional $GABA_BR$ heterodimers, and may suggest the existence of an alternative assembly of $GABA_BR1$ with other protein partners (Calver et al., 2000).

In the lens, the $GABA_AR$ (and probably $GABA_BR$) 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). In the adult brain GAD65 is greatly enriched in the presynaptic terminals and is thought to contribute mainly to the synaptic GABA pool (Battaglioli et al., 2003). Our data strongly suggest that in the embryonic lens GABA synthesized by GAD65 is released predominantly by VGAT and binds to $GABA_AR$ $\alpha_{2,3}$ subunit-containing receptors, while GABA produced by GAD67 is released predominantly by the membrane transporter GAT2 and binds to α_1-containing $GABA_AR$. GABA made by the enzymatically active GAD44, which is co-expressed with GAD65 (correlation coefficient 0.94) and is the embryonic counterpart of GAD67 (Szabo et al., 1994) may be released by inversion of one or more membrane GATs, as previously suggested (Szabo et al., 1994; Varju et al., 2001).

The four known membrane GABA transporters (GAT1-4) expressed in both glia and neurons can mediate GABA re-uptake from the extracellular space, but can also release GABA in exchange for 2Na+ and $1Cl^-$ (Jursky and Nelson, 1996; Richerson and Wu, 2003; Wu et al., 2007). The high level of expression of GAT1-4 showing overlapping temporal profiles and especially the robust expression of GAT4 suggests that GAT-mediated GABA uptake and release coupled with Na^+/Cl^- transport perform important function in the lens. 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 (Schouboe et al., 2004). The developmentally earlier GAT3 is sorted to the apical membranes of polarized lens epithelia and apical/lateral membranes of differentiating lens fibers compared to the postnatal GAT2, which is sorted predominantly to the basolateral

membrane (Muth et al., 1998). In comparison, the axonal GAT1, localized preferentially to apical membranes of polarized epithelium (Muth et al., 1998) and apical/basal surfaces of lens epithelium and fibers may carry out GABA transport at the fibers tips at stages of highest GABA production (E17.5-P0). The GABA transport across the lateral membranes is probably carried out by GAT3 during prenatal and GAT2 during postnatal stages. Finally, the predominantly radial glia-specific GAT4 (Jursky and Nelson, 1996) is pharmacologically unique among all transporters since it can operate as a Cl^- channel in the absence of Na^+ (Karakossian et al., 2005). Further experiments will be needed to clarify the possible roles of the abundantly expressed membrane GABA transporters in the lens ion transport and fiber differentiation.

The vesicular transporter VGAT mediates GABA (and glycine) transport into synaptic or synaptic-like vesicles used for GABA release from GABAergic cells of the nervous system and periphery (Chaudhry et al., 1998; Gammelsaeter et al., 2004; Wojcik et al., 2006). In the lens VGAT is more abundant before birth and in comparison with GATs, it was highly enriched at apical/basal cell membranes, but was never detected on the lateral surfaces. The high accumulation of VGAT in lens cells may suggest its specific role in vesicular GABA transport.

GAD, GABA, VGAT and GABAR show similar expression profile to some typical neuronal synaptic vesicular transport-related proteins like synapsin, synaptotagmin, synaptophysin (Frederikse et al., 2004; Min et al., 2004) and Snap-25 (Wride et al., 2003) in the developing lens, which suggests the existence of a GABA signaling system similar to the synaptic GABA transmission in the nervous system. Vesicles and microtubule arrays have been found to be particularly abundant in the elongating lens fiber cells (Lo et al., 2003). Like synapsin, VGAT, $GABA_AR$ and $GABA_BR$ are present in clusters at the tips of elongating fibers and apical membranes of the lens epithelium reminiscent of focal regions where vesicles interact with the fiber cell surface.

4. GABA receptors are functional in the developing lens: activation of GABA receptors triggers rise of intracellular calcium levels

It is now well established that GABA, by activating both $GABA_A$ and $GABA_B$ receptors, exerts a variety of trophic effects in the developing brain. Several types of $GABA_A$ receptor subunits have been described as components of functional GABA receptors in rat neuroepithelial cells, neuroblasts and glioblasts during spinal and neocortical neurogenesis, through which GABA has an effect on cell proliferation, migration, differentiation and

survival (LoTurco et al., 1995; Ma and Barker.,1995; Ma et al.,1998; Serafini et al.,1998a, 1998b; Verkhratsky and Steinhauser., 2000). GABA receptors are also expressed on non-excitable cells, and GABA acts as a trophic factor during the development of not only brain structures (Barker et al., 1998; Owens and Kriegstein, 2000a; 2000b; Varju et al., 2001; Watanabe et al., 2002; Represa and Ben-Ari, 2005) but also non-neuronal structures, like pancreas, testis and growth plate chondrocytes (Gu et al., 1993; Geigerseder et al., 2003; Franklin and Wollheim, 2004; Cheng et al., 2007). In these structures GABA induces $[Ca^{2+}]_i$ rise either from internal stores or through voltage gated Ca^{2+} channels. It has been previously revealed that lens cells also have a critical dependence on Ca^{2+} signaling for survival and growth (Duncan et al., 1997).

The activation of $GABA_A$ receptor triggers a depolarization-evoked Ca^{2+} influx through L-type VGCC resulted in increased intracellular calcium level, which in turn initiates a Ca^{2+}-dependent intracellular signaling cascade. Calcium as an intracellular second messenger (or signal) controls a wide range of cellular processes such as cell growth and survival, cytoskeleton remodeling, cell adhesion and gene regulation (Berridge et al., 2000).

$GABA_B$ receptors are G-protein-coupled and mediate the GABA signal via several second messenger pathways including calcium (Hagiwara et al., 2003). The presence of functional $GABA_B$ receptors in the developing cerebral cortex was also confirmed (Janigro and Schwartzkroin., 1988; Cherubini et al., 1997; Fukuda et al., 1993), and pharmacological studies suggested that through their activation GABA act as a chemoattractant to the immature cortical neurons.

We detected a variety of $GABA_A$ and the two $GABA_B$ receptor subunits in the developing lens that could form functional receptors. Based on the assumption that during development GABA acts through depolarization evoked calcium influx, we tested whether GABA receptor activation triggers intracellular calcium rise in developing lens.

As expected, we could really detect simultaneous rise in $[Ca^{2+}]_i$ upon GABA application in epithelial cells and elongating fibers in the equatorial region of freshly isolated P0 lenses loaded with the calcium sensitive dye Fluo-3/AM, indicating the existence of active GABA signaling in this region. The responding cells may correspond to the population of chicken annular pad cells responding to muscarinic acetycholine receptor stimulation with a $[Ca^{2+}]_i$ rise (Oppitz et al., 2003).

Surprisingly, in primary lens epithelial cell cultures, in which we also detected the molecular components of GABA signaling, both $GABA_AR$ agonist muscimol and $GABA_BR$ agonist baclofen evoked Ca^{2+} transients, predominantly in lentoids and sheets of epithelial

cells that could be blocked by the selective antagonists bicuculline and CGP55845, respectively. The $GABA_AR$-mediated Ca^{2+} rise in LEC is consistent with the channel properties during embryonic development (Barker et al., 1998; Owens and Kriegstein, 2002b) and/or non-neuronal cells (Watanabe et al., 2002; Franklin et al., 2004). On the contrary the $GABA_BR$-evoked calcium responses were unexpected, as $GABA_BR$ activation does not course membrane depolarization and therefore it is not linked directly to voltage gated calcium channels (Kerr and Ong, 1995; Couve et al., 2000; Owens and Kriegstein, 2002b; Watanabe et al., 2002; Lujan et al., 2005). However, more recent reports indicate that activation of $GABA_BR$ can induce Ca^{2+} rise in both neurons (De Erausquin et al., 1992; Guatteo et al., 2004) and astroglia (Meier et al., 2008). In the latter case, the atypical $GABA_BR$ responses displayed a variable and transient character mediated by Ca^{2+} release from IP_3- sensitive intracellular Ca^{2+} stores. Similarly we have found a greater variability in the LEC responses to baclofen compared to muscimol (data not shown), which indicates a dependence on threshold mechanisms. Alternatively, this variability could arise also from the superimposed inhibitory and excitatory effects of $GABA_BR$ existing in different cells (data not shown). However, the inhibitory component is probably small compared to the excitatory one, since application of the antagonist (CGP55845) alone usually had no effect, but occasionally evoked long-lasting oscillations, the mechanism of which is obscure.

The GABA signaling described here is a novel signaling system for the lens in addition to the previously characterized acetylcholine signaling (Duncan and Collison, 2003), which is involved in controlling cellular movements during lens development (Oppitz et al., 2003; Duncan and Collison, 2003). Based on previous and our (Oppitz et al., 2003) expression studies, the two systems may operate in the same epithelial and fiber cells to induce rise of $[Ca^{2+}]_i$ via GABAR and/or muscarinic receptors, respectively. Synergistic action of GABA and acetylcholine has been described previously for the chick retinal ventricular zone, where purinergic, muscarinic, GABAergic and glutamatergic stimulation all induce $[Ca^{2+}]_i$ rise (Pearson et al., 2002). Neurotransmitter co-expression and co-release is thought to be an important feature of the neuronal progenitors that may provide for compensatory mechanisms during early development (Owens and Kriegstein, 2002a; Demarque et al., 2002).

5. Possible functions of the GABA signaling in the developing lens, based on characterization of spatio-temporal expression of the molecular components

Structurally, GABA synthesis (GAD), release/uptake (VGAT, GAT) and GABA binding (GABAR) are co-localized in epithelial and immature lens fiber cells that allow

epithelial-to-epithelial, epithelial-to-fiber and fiber-to-fiber GABA-mediated signaling (Fig. 33).

Our results show that cells located in the germinative and migratory zones of the lens epithelium and elongating primary or secondary fibers express a functional GABA signaling and therefore GABA may act in a paracrine/autocrine fashion to modulate the cell cycle, cell migration and fiber elongation through modulation of the $[Ca^{2+}]_i$ level, by analogy with its similar role in the ventricular (germinative) and subventricular zones of the embryonic nervous system (LoTurco et al., 1995; Barker et al., 1998; Haydar et al., 2000; Owens and Kriegstein, 2002b). Both VZ cells and lens epithelial cells are polarized epithelia connected via gap junctions that are permeable for Ca^{2+} signal propagation upon activation of the GABA receptors.

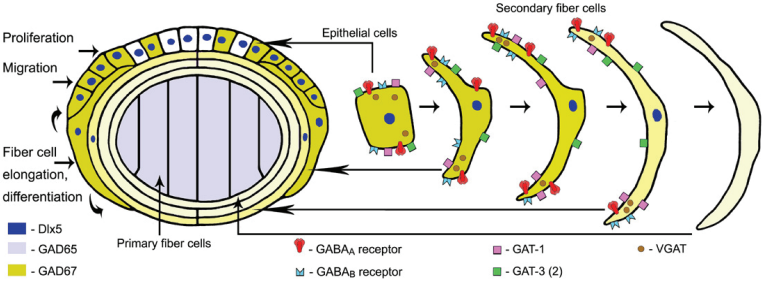

Fig. 33. Expression patterns of different GABA signaling components in the neonatal mouse lens: a schematic presentation. GAD65 is localized almost exclusively in the primary lens fibers comprising the lens nucleus, while GAD67 is mostly found in the elongating secondary fibers and lens epithelium cells. Dlx5, an upstream regulator of GAD genes is expressed in the nuclei of the epithelial cells and fibers expressing GAD and GABA. $GABA_A$ and $GABA_B$ receptor subunits, VGAT and GAT1 show preferential expression in the apical/basal membranes (including the lens sutures) of both lens epithelial cells and elongating secondary fibers at the equatorial region. GAT3 is expressed in the apical/basal membranes of epithelial cells and the apical and lateral membranes of fiber cells, but not expressed in the basal tips of elongating secondary fibers. GAT2 may be predominantly localized to lateral membranes (see Discussion).

The fiber cell differentiation in the lens is thought to proceed through an unusual, attenuated apoptosis (Ishizaki et al., 1998; Wride, 2000; Bassnett, 2002; Weber and Menko, 2005; Zandy et al., 2005), a Ca^{2+}dependent process during which the lens loose all membrane organelles including the nuclei. GABA and GABA signaling components, initially highly expressed in nucleated immature fibers are gradually lost and become undetectable in terminally differentiated enucleated fibers implying that the GABA signaling may be one of the regulators of this process, acting through $[Ca^{2+}]_i$ changes (Fig. 33). The spatio-temporal segregation of the components downstream of the two kinetically different GAD forms- the predominantly embryonic GAD65 (and EGAD) vs postnatal GAD67 is probably related to the profoundly different mechanisms of denucleation of the primary vs secondary fibers (Wride,

2000).

Furthermore, GABA synthesized by GAD65 and EGAD may be released by VGAT at the apical/basal end or membrane GAT1-3 at both apical and basolateral membranes, while GAD67- produced GABA is released almost entirely by GAT2/3 at apical/basolateral surfaces of epithelial and fiber cells (Fig. 33). Therefore, GABA pools synthesized by different GAD enzyme may influence differentially the intercellular communications through adherens and gap junctions and/or reorganization of the membrane skeleton, both Ca^{2+}-dependent processes as discussed in Kwakowsky et al., 2007.

The selective membrane sorting of different GATs to apical-basolateral surface of polarized cells is well documented (Muth et al., 1998; Schousboe et al., 2004) and may be related to their active role in the ion exchange, and regulation of the ion homeostasis and cell volume (Schousboe et al., 2004). A similar, yet unrecognized role for the membrane GATs can be proposed for the lens on the basis of their abundant expression, specific temporal regulation and selective membrane targeting at stages of most intensive fiber elongation.

Finally, the existence of GABA signaling machinery in the developing lens would imply that it could be a target to a variety of drug treatments acting predominantly on GABAR and transporters (Hadjikoutis et al., 2005) and also in autoimmune diseases affecting GAD, like IDDM (Freel et al., 2003). Since the lens cataract is a leading cause for blindness, it would be important to study the effect of such treatments or pathological conditions on the lens transparency in order to develop preventive strategies.

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

6. 1. Knockout versus overexpression?

The ability to engineer the mouse genome has proven useful for a variety of applications in research, medicine and biotechnology. Transgenic mice have become powerful tools for modeling genetic disorders, understanding embryonic development and evaluating therapeutics. These mice and the cell lines derived from them have also accelerated basic research by allowing scientists to assign functions to genes, dissect genetic pathways, and manipulate the cellular or biochemical properties of proteins. Modifying the expression of a gene in vivo, and the analysis of the consequences of the mutation, are central to the understanding of gene function during development and physiology, and therefore to our understanding of the gene's role in disease states.

Gene targeting techniques have revolutionized the field of mouse genetics and allowed

the analysis of diverse aspects of gene function in the context of the whole animal. Conventional gene targeting leads to inactivation, like in GAD65 knock-out and GAD67 knock-in mice, or modification of a gene in all tissues of the body from the onset of development throughout the whole lifespan. More recently, conditional gene targeting approach have been developed aimed at controlling gene targeting in a time- and tissue-dependent manner. This method is particularly useful in cases where complete gene inactivation leads to a lethal (GAD67 knock-in) or otherwise adverse (GAD65 knock-out) phenotype that prevents a more detailed analysis. Control of gene targeting in a time dependent manner allows the differentiation between effects of chronic versus acute depletion of a protein and also the analysis of functions at different time points in development. Many knockout mice revealed an unexpectedly minor phenotype that was attributed either to gene redundancy, or to adaptive mechanisms mediating developmental plasticity. Gene inactivation at a specific time point in the adult, leaving gene function intact throughout ontogeny, should prevent these adaptive responses and therefore phenotypes are expected to be more severe in conditional, as opposed to conventional, knockout mice. Moreover, if a given gene has a widespread pattern of expression, tissue-specific gene inactivation may define physiological roles of the gene product in a certain tissue, without compromising other functions in the organism (Muller, 1999).

An other effective way to study gene function in the mouse is overexpression of a protein of interest in a tissue specific way. The transgene encoded protein expression could be quite high due to multiple copies of the integrated transgene. The observed phenotypes are different from that of gene inactivation and sometimes are even more pronounced, but the main advantage of this method is that compensation is rarely observed. This technique is relatively quick; therefore disadvantages like the effect of integration site on transgene expression could be overcome by creating and analyzing several independent lines.

Even when the knockout mutant has a clear-cut phenotype, overexpression can still be valuable because it can generate completely unexpected phenotypes and thus shed light on aspects of protein function that would be missed otherwise. It can be particularly reassuring to confirm that the correct protein function has been identified when the overexpression phenotype is the opposite of the knockout phenotype, although it is still not clear how common this is (Zhang, 2003).

To study in vivo the role of GABA signaling in the developing lens, for the above reasons, we generated transgenic mice that overexpress GAD67 specifically in the lens and compared the resulted phenotype with that of mouse models lacking GAD65 and/or GAD67.

6. 2. Altered GABA signaling in human genetic diseases and in genetically modified mouse models causes multiple disorders

Genetic studies show that altered GABA levels can have severe consequences on development of both human and mice. In humans, genetic mutations that disrupt the SSADH, enzyme of the GABA shunt, or the GABA degrading enzyme GABA-T causes severe clinical manifestations associated with the accumulation of neurotransmitters in physiological fluids (Chambliss et al., 1998; Medina-Kauwe et al., 1999). In addition, another genetic disease characterized by generalized seizures in the first hours of life is thought to be linked with a deficiency in GAD (Cormier-Daire et al., 2000).

Genetic modification of GABA signaling in mice has demonstrated that GABA plays important roles in the adult and developing CNS and also in the development of non-neuronal tissues. Knockouts of both GAD67 and $GABA_A$ receptor subunit β3 lead to cleft palate and early neonatal lethality (Asada et al., 1997; Homanics et al., 1997). GAD65 knockout mice show epilepsy, increased anxiety, increased sensitivity to benzodiazepines, and impaired developmental plasticity in the cortex (Kash et al., 1997; Hensch et al., 1998; Kash et al., 1999). Transgenic mice overexpressing GAD67 exclusively in the gonadotropin-releasing hormone secreting neurons clearly showed that GABA is involved in the regualation of migration of these neurons (Heger et al., 2003).

6. 3. GABA signaling is required for the proper regulation of cell proliferation and fiber cell differentiation

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) events, which occur throughout life. Distinct cellular processes during lens development and growth are dependent on the tight spatial and temporal regulation of epithelial cell proliferation and their differentiation into fiber cells controlled by numerous transcription factors and growth factor-induced signaling pathways.

As we have demonstrated, one of the factors involved in regulation of these processes is GABA.

Analysis of lens development in mouse models overexpressing GAD67 or lacking GAD65 and/or GAD67 cleraly showed that GABA is essential for multiple developmental events. It plays an important role at late embryonic and early postnatal stages in the

elongation/differentiation of the primary fiber cells and in the proliferation of the anterior lens epithelium that forms the secondary fiber cells. GABA may also be required for proper cell adhesion, a key process for establishing appropriate lens structure.

6. 3. 1. GABA is essential in the regulation of primary fiber cell elongation

During normal mouse lens development after the formation of the lens vesicle, the mitotically active epithelial cells from the anterior pole are induced to differentiate into fiber cells in the equatorial region encircling the lens. The fiber cells elongate anteriorly to fill the hollow lens vesicle, eventually producing a lens consisting of postmitotic differentiated fiber cells covered anteriorly by a monolayer of proliferating epithelial cells. Although the initial induction of the lens vesicle was unaffected in genetically modified mouse models, either overexpressing GAD67 in the lens or carrying inactivated GAD genes, there was a defective elongation of the primary lens fibers towards the anterior epithelium. During lens vesicle stage (from E12.5), epithelial cell elongation was accelerated in lenses overexpressing GAD67 and was delayed in GAD65-/- and GAD65-/-; GAD67-/- mice, while primary fiber cell elongation in GAD67-/- mice was not affected.

Our data indicate, that a precisely regulated GABA level is critical for proper primary fiber cell elongation. Furthermore, based on the phenotypic analysis of mutant lenses and the expression analyis of different GADs in the developing lens, we suggest that GABA synthesized by GAD65 may influence the elongation process since it is the most abundant GAD form at this developmental stage.

6. 3. 2. GABA as a modulator of lens epithelial cell proliferation

In the mouse lens vesicle (at E11.0–E11.5), nearly all cells have the capacity to proliferate, but some in the extreme posterior may have already begun to withdraw from the cell cycle. By approximately day E13.0, primary fibers have occluded the lens vesicle and proliferation is restricted to the anterior epithelium, as the primary fiber cells and cells in the transition zone have withdrawn from the cell cycle permanently. After birth, the region of high proliferative activity is restricted to the germinative zone whereas newly differentiating secondary fibers in the transition zone are postmitotic. In the central epithelium, most cells are in a quiescent state, although few proliferating cells are present (for review see Griep, 2006).

The strong epithelial expression of GAD67 in the germinative zone suggests that GABA synthesized by this form might be involved in the regulation of the second wave of cell proliferation in this region. Until approximately E15.5 the rate of proliferation is similar

in the wild type and mutant lenses, but in the absence of GAD67, cell proliferation rate is reduced from embryonic day 16. On the contrary, overexpression of GAD67 leads to increased proliferation in the lens epithelium from E17.5. As a result of this abnormal process, the cells of the anterior lens epithelium do not form a single layer but, rather form multilayered structures that do not properly separate from the cornea. In GAD65-/- mice proliferation of epithelial cells is not affected, because this isoform was not expressed in lens epithelial cells during this period.

The ratio of the lens fiber cells to anterior lens epithelial cells is carefully controlled by the rate of proliferation and differentiation. It is known that certain growth factors such as PDGF stimulate the proliferation of cultured lens epithelial cells (Brewitt and Clark, 1988) and that overexpression of PDGF (Reneker and Overbeek, 1996) and IGF (Shirke et al., 2001) in transgenic mice leads to increased levels of proliferating cells in the epithelium. Initial studies are indeed now beginning to shed light on the important players in cell cycle control in the epithelium, but details of the mechanisms still remain to be elucidated.

Our data suggests that similarly to the developing nervous system, GABA synthesized by GAD67 might be a modulator of epithelial cell proliferation in the lens from late embryonic stages to adulthood.

6. 3. 2. 1. Development of retina and anterior ocular tissues is affected in transgenic mice with altered GABA level

We did not see difference between wild-type and mutant anterior germinal epithelium at E10.5-E15.5. However, from E12.5 we found abnormal proliferation in the retina of transgenic mice overexpressing GAD67 in the lens. This could be the consequence of elevated GABA level either in the lens or retina, since low-level of ectopic transgene expression in the retina cannot be ruled out (data not shown; Xi et al., 2003). The GAD67-/- eye was smaller than wild-type, due to reduced proliferation of neural retina. For the same reason, we detected similar cell proliferation defects in anterior eye structures, such as the iris, cilliary body and pigment epithelium.

As a consequence of increased cell proliferation in CrysGAD67 transgenic mice, the retina displayed abnormal foldings and ectopic retina also developed. A number of ocular defects in transgenic mice that overexpress GAD67, such as fusion of the lens to cornea, iris or cilliary body, could be the secondary consequence of retinal over-proliferation. Since the congenital retinal folds may have pushed the lens against the cornea, resulting in mechanical stimulation or injury of the lens epithelium (Gorthy, 1979).

However, the lack of separation of the anterior lens epithelium from the cornea, and the lens-cilliary body, lens-iris, lens-retina fusions might also be related to the abnormal expression of cell adhesion molecules such as the N- and E-cadherin, as altered expression of these adhesion molecules were also demonstrated in the CrysGAD67 lens.

The above disorders in the development of the retina and anterior ocular tissues are most likely secondary defects caused by lens abnormalities. Proper eye formation depends on specific interactions between lens, retina and cornea coupled with temporally distinct gene expression and a regulated sequence of signaling events. However, the place and exact role of GABA in this complex signaling network has not been resolved yet.

6. 3. 3. GABA is required for the proper differentiation of lens fibers

A major consequence of fiber terminal differentiation is the transformation of selected cuboidal lens epithelial cells, <5 μm high, into the ribbon-like fibers that range in length from 150 μm to 2.5 mm in the mouse lenses. The creation of these exceedingly long fibers is characterized by the elaboration of specific cytoplasmic and membrane proteins: the crystallins (Halder and Crabbe, 1984) and major intrinsic protein (MIP), respectively. During their differentiation, fiber cells become progressively longer until their tips reach the sutures at the anterior and posterior poles of the lens. At the sutures, they contact fiber cells from the opposite side of the lens. During differentiation, all fiber ends must reach a precise migratory destination for the purpose of forming and maintaining a particular suture pattern. Improper or disorganized fiber end migration leads to the formation of irregular and/or excess suture branches, reduced lens optical quality (Kuszak et al., 1991, 1994; Sivak et al., 1994), and cataract (Kuszak et al.,1994; Al-ghoul et al., 1998). During elongation, fiber cells maintain contact with the epithelial layer and lens capsule, respectively, through interactions involving the tips of the fiber cells (Bassnett et al., 1999, 2003). Anterior fiber ends migrate along the apical surface of the epithelium, while posterior fiber ends migrate along the inner surface of the capsule. When elongation is completed, the ends of lens fibers detach from the epithelium or capsule, and then abut with opposing fibers to form the lens sutures. Although the mechanisms controlling the rate of migration along these substrates is not understood, recent evidence suggests that complexes (BMC-basal membrane complex) involving the basal domain of the fiber cell membrane, its integral membrane proteins (integrins, N-cadherins) and their associated cytoskeletal elements (actin bundles), facilitate interactions with the capsule (Bassnett et al., 1999).

The preferential accumulation of the “early” GADs and other GABA signaling

components at the apical and basal tips of the lens fibers, which are the sites of active remodeling of the membrane skeleton during fiber elongation (Lee et al., 2000), indicates that GAD65 and EGAD and ultimately GABA may play a role in this process. Furthermore, the "early" GADs through GABA production may be involved in cell signaling during formation of transient cell-adhesion complexes between elongating fiber cells and overlying lens epithelium and/or the lens capsule at the posterior lens wall. Similarly, the extremely high levels of accumulation of GAD, GABA receptors and transporters at the anterior and posterior lens poles and the conspicuous "patchy" GAD staining in the central lens epithelium during E13.5-E16.5 suggest that GAD and GABA are involved in cell adhesion mechanisms between fibers of opposite sides during formation of lens sutures (Fig. 33) (Beebe et al., 2001).

Phenotypic analysis of adult CrysGAD67 transgenic lenses also supports to the above hypothesis, since lenses with elevated GAD/GABA levels displayed deformed fiber tips and lens sutures**.**

Sutural defects, like in CrysGAD67 trangenics, may occur when the elongation, migration, and detachment of fiber ends is disrupted (Kuszak et al., 2004). At the termination of their migration, basal fiber ends detach from the capsule and interdigitate to form the suture branches. Improper or disorganized fiber end migration leads to the formation of irregular and/or excess suture branches that become aligned along the visual axis. This excessive disorganization has been shown to adversely affect lens optical quality and lens transparency (Hanna and O'brien, 1963; Shiels et al., 2000; Moré et al., 2001; Xia et al., 2006). Sutural defects are commonly associated with specific types of cataract (Bassnett et al., 1999; Zhang et al., 2004). Recently, human sutural cataracts have been associated with mutation in BFSP2, the gene encoding beaded filament structural protein 2 (Conley et al., 2000; Jakobs et al., 2000; Zhang et al., 2004).

In tissues, there is often a close connection between structure and function. This is particularly true for the lens where maintenance of its unique cytoarchitecture is crucial to the preservation of its transparency. Previous studies have demonstrated that the establishment of normal lens cell morphology is dependent on the assembly of cadherin-based cell-cell junctions (Ferreira-Cornwell et al., 2000). In GAD67 overexpressing lenses these adherens junctions are disrupted, linearity of fiber cell membranes is lost and fiber cell are separated, defects common to many types of cataract (Bettelheim et al., 1997; More, 2001; Frederikse, 2002).

Opacification of the lens is typically caused by light scatter resulting from morphological defects in the lens fiber zone, but there is little understanding of the early

morphogenetic changes associated with cataractogenesis or altered morphogenetic pathway in the fiber zone that leads to the development of cataract. The membrane defects are the earliest morphological defects reported for lens fiber cells during cataractogenesis. The opacification itself is due to the disorganization of fiber cells.

We detected marked disruption of both cortical and nuclear fibers in Crys GAD67 mice, indicating that both primary and secondary fiber cells are affected. As a consequence, the eyes of CrysGAD67 mice showed a weak but recognizable cataract, characterized by visible opacity in the CrysGAD67 lens, which was enhanced in aged mice.

The exact mechanisms of cataract formation are not known. Several forms of cataracts are connected to altered epithelial and fiber cell differentiation resulting in structural and functional abnormalities of the lens (Hightower et al., 1994; Wegener, 1995; Ruiz-Ederra and Verkman, 2006; Bu et al., 2002). A growing number of genes and signaling molecules are suggested to be be involved in this process, and as we demonstrated GABA could be one of these candidates (Harding, 1991).

7. Signaling factors during lens development: the role of GABA

Since the lack and overexpression of GAD both leads to defects in lens development that cannot be attributed to only one specific process, it appears that different GAD isoforms through functionally distinct GABA pools are integral components of a regulatory network, which controls the proliferation and differentiation of lens cells. Several genes act in concert in this process, therefore it is difficult to determine the exact role of single molecules.

The identification of human mutations associated with lens defects in conjunction with biochemical and genetic studies in mice have allowed the identification of a number of transcription and growth factors, and cell cycle regulatory proteins that play important roles in normal lens formation. Thus, for example, transcription factors Pax-6, L-maf, c-maf, and Sox1 have been shown to play important roles in eye development. Pax-6 (in the mouse) (Grindley et al., 1995; Hanson and Van Heyningen, 1995) and L-maf (in the chicken) (Ogino and Yasuda, 1998) appear to function as master regulators, as animals lacking these factors fail to develop eyes. c-maf, Prox1 and Sox1 are required for elongation of the primary lens fibers during early lens development (Nishiguchi et al., 1998; Kawauchi et al., 1999; Kim et al., 1999). Transgenic and gene disruption studies have provided evidence for the capacity for several growth factor signaling pathways to affect lens cell proliferation and differentiation. Growth factors including FGF-1 and FGF-2 (McAvoy and Chamberlain, 1989), PDGF (Kok et al., 2002), EGF/TGFα (Wang et al., 2005), and HGF (Wormstone et al., 2000; Choi et al.,

2004) all have been shown to stimulate lens epithelial cell proliferation in a variety of species (for review see Lovicu and McAvoy, 2005). On the other hand, TGF_{β} has been shown to inhibit the proliferation of human lens epithelial cells in culture (Wormstone et al., 2004). Growth factors BMP-7 (Dudley et al., 1995; Luo et al.,1995), FGF (Robinson et al., 1998) and IGFs (Arnold et al., 1993; Beebe et al., 1987) all appear to regulate the differentiation and elongation of the lens fiber cells.

Based on our findings, here we propose a model for integrating GABA into the signaling network to regulate lens development (Fig. 34).

Our data indicate that GABA is involved in trophic signaling from early stages of the lens development, probably as a modulator of lens epithelial cell 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.

Analysis of transgenic eyes with enhanced GAD67 expression from early stages of development indicates that even low-level expression of the CrysGAD67 transgene, which corresponds to small elevation in GABA level, can have a profound impact on lens morphology. However, using a stronger early promoter (like Pax6) might be a better choice to overexpress GAD, since we could obtain data about GABA function from earliest stages of development. Furthermore, lens-specific (epithelial and fiber cell specific) GAD knock-out mice (for example crossing Pax6-Cre and GAD67floxed mice) could also provide very important information on the exact role of GADs and GABA in different compartments and developmental stages of lens development.

Further studies are needed to determine the exact mechanism of GABA action during cell proliferation/differentiation both in the lens and CNS, which may yield important insights into novel therapeutic approaches to treat or prevent diseases that cause blindness and CNS disorders.

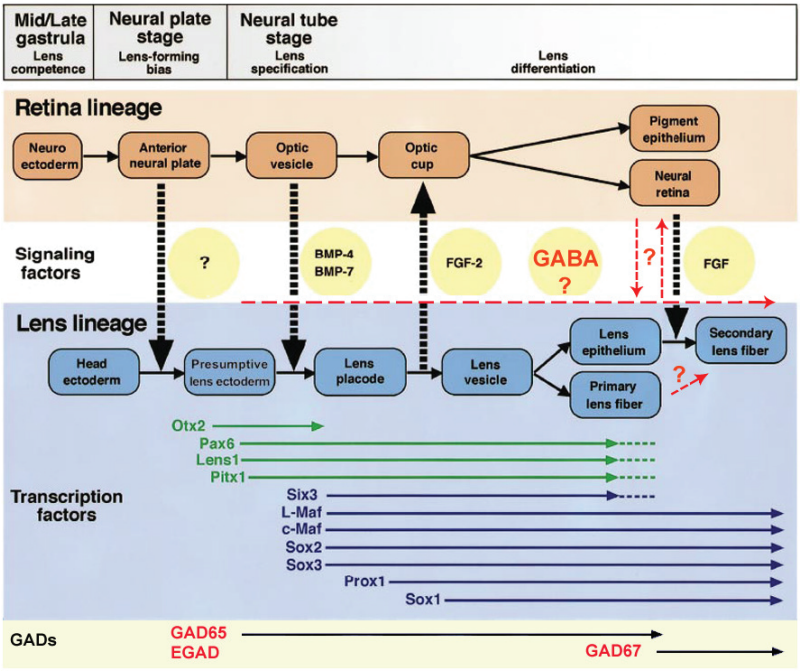

Fig. 34. Sequential activation of transcription- and signaling factors during lens development. Diagram showing the link between inductive interactions, expression of transcription factors during lens development as well as the possible role of GABA and different GAD form in this process. Developmental stages (at the top) and major inductive events between the retina- and lens-forming tissues (bold, dotted arrows) are illustrated according to the currently favored model (Grainger 1992; Pittack et al., 1997). Signaling factors implicated in the respective inductive events are shown in yellow circles. The green and blue arrows represent the expression profile of transcription factors that were analyzed mainly at the messenger ribonucleic acid (mRNA) level in Xenopus, chicken, and/or mouse embryos. Only factors whose expression has been fairly well characterized are shown. The black arrows at the bottom represent the expression profile of GADs. GABA might be involved in the regulation of epithelial cells proliferation and fiber cell differentiation (red broken arrows). Modified from Ref.: *Ogino and Yasuda, 2000.*

VII. Summary of major findings

- We showed for first time that GABA and all forms of its biosynthetic enzyme GAD 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 in shaping of the cytoskeleton during fiber elongation stages.
- We also demostrated that Dlx2 and Dlx5 transcription factors are expressed sequentially in the developing lens. Dlx2 expression is 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.
- 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, and characterized their temporal and spatial expression during development.
- 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 cultures, demonstrating the presence of functional GABA signaling in the developing lens.
- 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, indicating that the 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.

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