Molecular components and functionality of the GABA signaling during development: a study on two model systems

Ph.D. Dissertation

Marija Schwirtlich

Semmelweis University

John Szentágothai School of Ph.D.Studies

Supervisor: Szábo Gábor M.D., Ph.D.

Institue of Experimental Medicine
Hungarian Academy of Sciences
Laboratory of Molecular Biology and Genetics

Scientific referees for the Ph.D.Dissertation:

Zsombor Lacza Ph.D.

Szente Magdolna Ph.D., D.Sc.

Committee Members at the Comprehensive Exam:

András Csillag Ph.D., D.Sc – Chairman

Èva Fekete Ph.D., D.Sc.

Zsuzsa Emri Ph.D.

To my dear parents

I TABLE OF CONTENT

II LIST OF ABBREVIATIONS

AAH	artificial aqueous humor
ALP	alkaline phoshatase
AMPA	a-amino-3-OH-5-methylioxazolate-4-propionic acid
AMPA-R	AMPA-gated glutamate receptor channel
DAB	3,3'-diaminobenzidine
CNS	central nervous system
CP	cortical plate
E	embryonic day
EB	embryoid body
EGAD	embryonic glutamic acid decarboxylase
EPSC, EPSP	excitatory postsynaptic current, potential
ES cells	embryonic stem cells
FBS	fetal bovine serum
GABA	γ-aminobutyric acid
$GABA_A$ receptor	GABA-gated receptor channel (type A)
$GABA_B$ receptor	GABA-gated receptor channel (type B)
$GABA_C$ receptor	GABA-gated receptor channel (type C)
GAD	Glutamic acid decarboxilase
GAT (1-4)	GABA transporters type 1-4
HEPES	*N*-hydroxyethylpiperazine-*N'*-2-ethanosulfonic acid
IP3	Inositol-3-phosphate
iPS	induced pluripotent cells
IZ	intermediate zone
K^+	potassium ion

KCC1-3	potassium chloride co-transporter type1-3
LVA Ca2+ channels	low-voltage activated calcium channels
MZ	mantle zone
Na^+	sodium ion
NKCC1-3	Sodium potassium chloride co-transporter type 1-3
NMDA	*N*-methyl-d-aspartate
NMDA-R	NMDA-gated glutamate receptor channel
PAF	paraform-aldehyde
PBS	phosphate buffer saline
PCR	polymerase chain reaction
PSC	postsynaptic current
RT	room temperature
RT-PCR	reverse transcribed polymerase chain reaction
RYR	Ryanodine receptor
SB	standard buffer
SDS-PAGE	sodium dodecylsulphate-polyacrylamide gel electrophoresis
SVZ	subventricular zone
TBS	Tris buffered saline
VGAT	vesicular GABA transporter
VGCC	voltage-gated calcium channels
VZ	ventricular zone
Wt	wild type

"New idea: at first, they said, it can't be true; when it became obvious it was true, they said it wasn't important; and when its importance couldn't be doubted, they said, it wasn't new!"

Adopted from "How does a little acronym become a big transmitter?", Krešimir Krnjević, 2006

III INTRODUCTION

1. γ - AMINOBUTYRIC ACID (GABA) SIGNALING

γ-aminobutyric acid (GABA) is a nonprotein amino acid that is conserved from bacteria through yeast and plants to mammals where it can exert diverse functions (Roberts, 1974; Work and Dewey, 1953; Marcus and Halpern, 1969; Bouche et al., 2003; Bouche and Fromm, 2004).

In the adult vertebrate central nervous system (CNS), GABA is mostly recognized as the principal inhibitory neurotransmitter and as such plays a key role in modulating neuronal excitability. It is estimated that, depending on the brain region, 20 to 50 % of all central synapses use GABA as their neurotransmitter (Bloom and Iversen, 1971; Docherty et al., 1985). The heterogeneity in types of GABAergic synapses and interneurons revealed in the last decade (Freund and Buzsaki, 1996; Parra et al., 1998; Cossart et al., 2006; Dumitriu et al., 2007) indicates that an array of GABAergic signaling functions might exist in the brain. GABA is directly involved in a variety of biological functions such as locomotor activity, learning, reproduction, circadian rhythms as well as cognition, emotions and sleep (Agmo et al., 1987; Paulsen and Moser, 1998; Mintz et al., 2002; Leventhal et al., 2003; Terasawa, 2005).

Furthermore, in the immature nervous system GABA serves as excitatory neurotransmitter and acting in a paracrine way (reviewed in Owens and Kriegstein, 2002a, 2000b; Ben Ari et al., 2002, 2007). Membrane and intracellular calcium rise

induced by GABA-mediated depolarization, activates second messenger–intracellular cascades involved in neuronal migration, growth, differentiation, plasticity of synaptic connections and establishment and patterning of neural networks (Owens and Kriegstein, 2002a, 2002b; Ben-Ari et al., 2002, 2007).

Proper function of the GABAergic system is crucial for the performing of higher brain functions. Various psychiatric and neurological disorders such as anxiety, depression, epilepsy, schizophrenia, and autism are partly caused by dysfunction of the GABAergic transmission and imbalanced excitation vs inhibition in the developing and/or mature brain (Wong et al., 2003).

Besides its function in the CNS, GABA has been also suggested to be involved in physiology of glia cells, digestive tract, pancreas, liver, osteoblasts, and chondrocytes (reviewed in Watanabe et al., 2002) and in modulating the proliferation of various cancer cells (reviewed in Watanabe et al., 2006).

1.1. Basic properties of GABA signaling

Molecular components and GABA neurotransmitter system is summarized in ***Fig.1***.

In the mammalian brain, **GABA** is synthesized primarily from glutamate in a reaction that is catalyzed by two glutamic acid decarboxylase (**GAD**) enzymes, GAD65 and GAD67. GABA is loaded into synaptic vesicles by a vesicular neurotransmitter transporter (**VGAT)** and is released from nerve terminals by calcium-dependent exocytosis. However, nonvesicular forms of GABA secretion (for example, by reversal of the membrane GABA transporter GAT) have also been described and might be particularly important during development (see below). The effects of GABA can be mediated by the activation of either ionotropic $\mathbf{GABA_A}$ or metabotropic $\mathbf{GABA_B}$ receptors, which can be localized both pre- or postsynaptically.

GABA signals are terminated by reuptake of the neurotransmitter into nerve terminals and/or into surrounding glial cells by the plasma membrane GABA transporters (**GATs**). Subsequently, GABA is metabolized by a transamination reaction that is catalysed by the mitochondrial GABA transaminase (**GABA-T**).

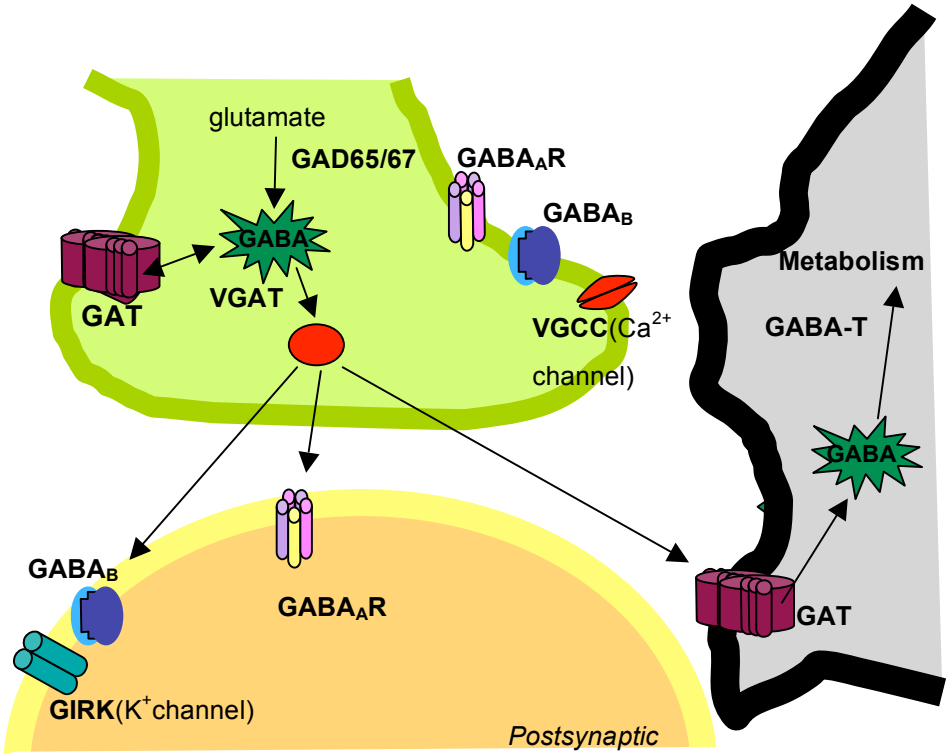


Fig.1. Molecular components of the GABA signaling. Schematic diagram of the synthesis and transport of GABA at synapses. GABA is synthesized primarly from glutamate in a reaction that is catalysed by GAD. GABA can be released either vesicularly (by VGAT) or nonvesicularly (by reverse transport). GABA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

1.1.1. GABA synthetic enzyme - the glutamic acid decarboxilase (GAD)

1.1.1.1. Two isoforms of GAD: GAD65 and GAD67

Following the successful purification of GAD in the laboratory of E. Roberts (Roberts, 1986), it has become possible to show that this enzyme catalyses the rate-limiting step in GABA synthesis from glutamate and that this is the predominate route for GABA synthesis in the CNS and periphery. These early studies have also shown that GAD is localized in the same neurons that use GABA and since then, it has been used as a morphological marker of GABAergic neurons.

Molecular cloning studies have shown that in the adult vertebrate brain GAD exists in two major isoforms, 65kD (**GAD65**) and 67kD (**GAD67**), which are products of two independently regulated genes located on chromosome 2 and 10, in humans and in the mouse, respectively (Soghomonian and Martin, 1998). Each of these forms is highly conserved among vertebrates and shares more then 95% identity at the amino acid level among different species such as rat, mouse, cat, and human (Kaufman et al., 1986; Kobayashi et al., 1987; Katarova et al., 1990; Wyborski et al., 1990; Erlander et al., 1991). In the same species, the two GAD proteins differ in amino acid sequence (with 65% identity in the rat), molecular sizes (65 kD and 67 kD), regional distribution in the brain, subcellular localization and regulation. Both forms are composed of two functional domains: a highly divergent N-terminal domain (with 23% identity between the two human GADs) that is involved in subcellular targeting, membrane association and heteromeric interactions, and a larger, more conserved (73% identity) C-terminal domain that contains the catalytic centre (Soghomonian and Martin, 1998; Varju et al., 2001). Experiments with recombinant GAD suggested that GAD65 is more responsive than GAD67 to the presence of the cofactor pyridoxal-P (PLP) (Erlander et al., 1991; Kaufman et al., 1991). This is consistent with the finding that the majority of apo-GAD (form without cofactor PLP) in brain is provided by GAD65 (Martin et al., 1991). Taking into account also the enrichment of GAD65 compared to GAD67 at synaptic vesicles of nerve terminals and its specific pattern of expression, GAD65 is thought to be preferentially responsible for the establishment of the vesicular GABA-transmitter pool. Thus this form is predominantly associated with stimuli-evoked, largely PLP-dependent inhibitory synaptic transmission. In contrast, GAD67 makes up most of the constitutively active holo-GAD (PLP bound GAD) pool and is enriched in the cytoplasm. The non-vesicular cytoplasmic GABA pool synthesized mostly by GAD67 is involved in tonic inhibition, which is less dependent on PLP availability

(Erlander et al., 1991; Martin and Rimvall, 1993). This form is mostly responsible for the synthesis of non-synaptic GABA pool that has metabolic functions (Martin and Rimvall, 1993; Esklapez et al., 1994; Soghomonian and Martin, 1998).

1.1.1.2. GABA metabolism

GABA is mainly metabolized through a short pathway called the "GABA shunt", a closed loop, which acts in such a way as to secure the supply of GABA (Bouche et al., 2003; ***Fig.2***). The first step in the shunt is the transamination of α-ketoglutarate to glutamic acid. Glutamic acid is then decarboxylated to form GABA. GABA is then transaminated to form succinic semialdehyde, but the transamination can only take place if α-ketoglutarate is the acceptor of the amino group. This transforms α-ketoglutarate into the GABA precursor, glutamate, thereby guaranteeing a continuity of supply. Thus, a molecule of GABA can only be degraded metabolically if a molecule of precursor glutamate is formed in its place. The succinid semialdehyde formed from GABA is rapidly oxidized to succinic acid, which is a normal constituent of the Krebs cycle.

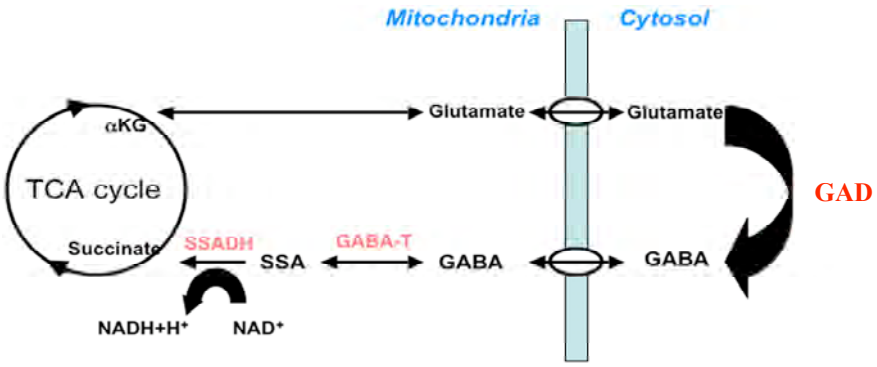


Fig.1. Schematic representation of the GABA shunt metabolic pathway. The GABA shunt is composed of three enzymes (red). Glutamic acid decarboxylase (**GAD**) is a cytosolic enzyme that catalyzes the irreversible decarboxylation of glutamate to produce GABA. GABA is transported into the mitochondria where it is converted into succinic semialdehyde (**SSA**) by GABA transaminase (**GABA-T**). **SSA** is then reduced by succinic semialdehyde dehydrogenase (**SSADH**) to form succinate, which enters the tricarboxylic acid (TCA) cycle. α-KG, α-ketoglutarate

Extensive studies on brain GABA have revealed the existence of two distinct GABA pools – synaptosomal, mediating synaptic inhibition and cellular, with a presumed

metabolic function (Martin and Rimvall. 1993; Martin and Barke, 1998). Roughly, less then 50% of neuronal GABA is engaged in the synaptosomal pool, consequently, there is a fairly large cytoplasmic GABA pool that could either be involved in a different functions, related to extrasynaptic cell-cell signaling or can serve as a reserve.

Finally, change in GABA homeostasis can have severe consenquences on development of both humans and plants (reviewed in Bouche et al., 2003). For example, in humans, disruption of SSADH or GABA-T causes severe clinical manifestations associated with the accumulation of neurotransmitters in physiological fluids (Chambliss et al., 1998; Medina-Kauwe et al., 1999).

1.1.1.3. Alternativey spliced truncated GAD embryonic forms

During embryonic development, two additional truncated GAD proteins: GAD25 and GAD44 are also expressed, which are the products of alternatively spliced GAD transcripts encoded by the GAD67 gene.

The two distinct embryonic GAD transcripts include two almost identical alternatively spliced exons inserted into the coding region of GAD67 (***Fig.3***; Bond et al., 1990; Szabo et al., 1994). The bicistronic I-80 mRNA contains the embryonic exon "7/A" (filled boxes, ***Fig.3***) with an overlapping translational stop-start codon (TGATG) and codes for both truncated enzymatically inactive GAD25 and the enzymaticallay active 44kD (**GAD44**) form, whereas I-86 mRNA containing the 86bp long exon "7/B" including an additional stop codon at the 3'-end codes only for GAD25 as the GAD44 open reading frame is interrupted in this message (***Fig.3***). GAD25 corresponds to the N-terminal regulatory domain of GAD67 and has been proposed to play mainly a regulatory role. GAD44 is roughly identical to the C-terminal catalytic domain of GAD67 and can synthesize GABA with putative developmental role (Szabo et al., 1994; reviewed in Varju et al., 2001).

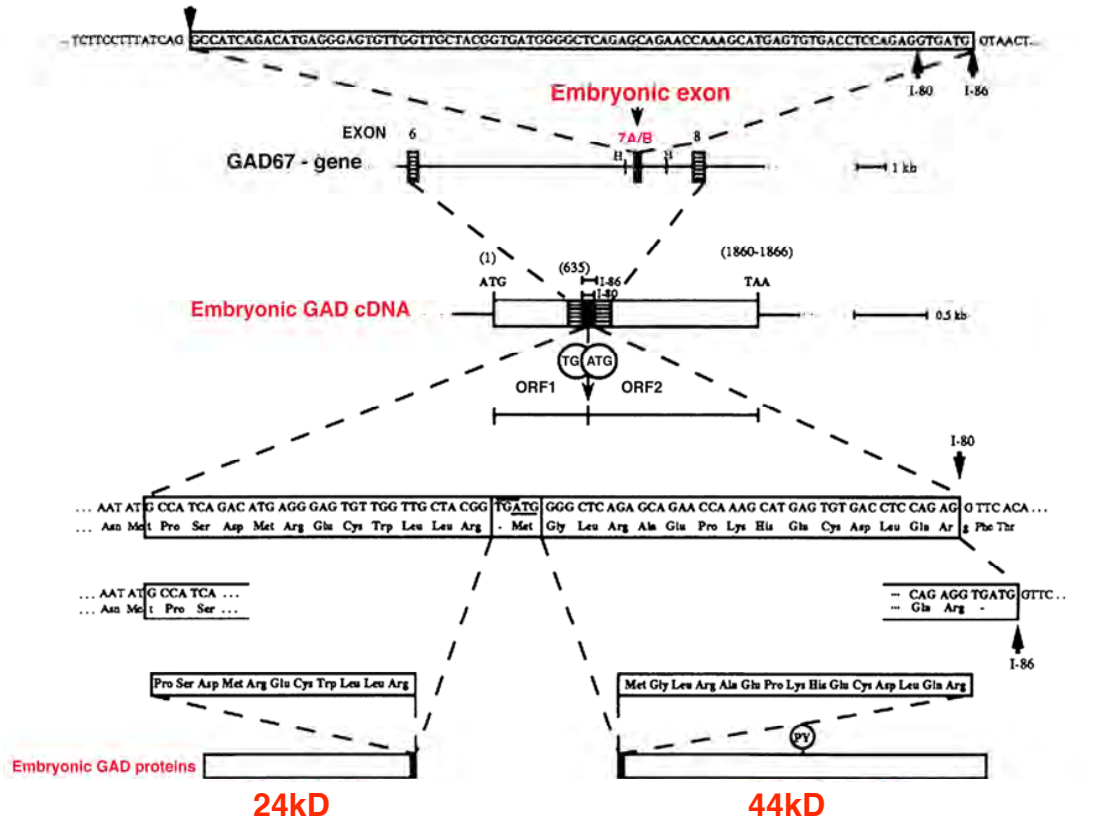


Fig.3. Schematic representation of the embryonic exon on GAD67 genomic and cDNA clones and the resulting putative protein products. GAD67 mRNA is rendered structurally and functionally bicistronic through the developmentally regulated alternative splicing of a single exon (7). The embryonic exon-derived TGATG overlapping codon splits the open reading frame of GAD67 into two (ORF1,2). Exons 6 and 8 are represented in striped boxes; The overlapping stop-start codon is circled. The corresponding amino acid sequences are given in a three letter code. Modified from *Szabo et al., 1994.*

1.1.1.4. Upstream regulators of GAD genes: the Dlx gene family

The *Drosophila melanogaster Distal-less (Dll)* gene codes for a homeodomain protein (Cohen et al., 1989) that is required for the proper development of the limb and for correct proximo-distal (PD) organization of the embryo (Cohen and Jurgens, 1989; Cohen et al., 1989). Distal-less related genes named Dlx in vertebrates code for transcription factors that show a high degree of sequence similarity in their DNA-binding domains and the homeodomains (Liu et al., 1997; Zhang et al., 1997). Dlx genes found in mice and man are found linked in a paired tandem convergent configuration, in a following order: Dlx1 and Dlx2, Dlx5 and Dlx6, Dlx3 and Dlx7, respectively. (Bulfone et al., 1993; Simeone et al., 1994; Stock et al., 1996; Anderson

et al., 1997a; Liu et al., 1997; Panganiban and Rubenstein, 2002; Zerucha et al., 2000). Recent work demonstrated that Dlx genes are expressed in spatially and temporally restricted patterns in craniofacial primordia, basal telencephalon and diencephalon, and in distal regions of extending appendages, including the limb and the genital bud (Merlo et al., 2000).

The expression pattern of Dlx genes in embryonic forebrain and some non-neuronal tissues is nearly identical to that of the GAD65 and GAD67 (Thomas et al.,1997; Katarova et al., 2000; Maddox and Condie, 2001; Depew et al., 2005; Tamayama et al., 2005; Levi et al., 2006). Forced expression of Dlx2 or Dlx5, but not Dlx1, in slice cultures of mouse embryonic cerebral cortex induced ectopic expression of GAD65 and GAD67 (Stühmer et al., 2002a) suggesting that Dlx2/5 are directly involved in activation of GAD genes and subsequently, in the establishment of the GABAergic phenotype (Stühmer et al., 2002a, 2002b).

1.1.2. Vesicular and membrane GABA transporters: GABA release and uptake

Following syntheis, GABA is loaded into synaptic vesicles by the vesicular GABA transporter (**VGAT**) directed by a Na^+-independent proton-electrical gradient, which is generated at the cost of H^+-ATPase. Upon neuronal stimulation GABA is liberated from the nerve terminals by calcium-dependent exocytosis (McIntire et al., 1997; Chaundry et al., 1998; Fon and Edwards, 2001). Similar mechanism is believed to apply to some non-neuronal GABA-synthesizing cells (Reetz et al., 1991; Geigerseder et al., 2003; Gammelsaeter et al., 2004), and indeed actual GABA release has been demonstrated recently in the β-cells of the pancreas (Braun et al., 2004).

The magnitude and duration of GABA action is dependent upon an effective mechanism by which the action of the transmitter can be cleared from the site of action. GABA is taken-up from the extracellular space by the membrane GABA-

transporters (GATs), members of the Na^+- and Cl^-- dependent transporters specialized in uptake of GABA in exchange for Na^+ and a Cl^--ions. To date four GATs have been cloned, GAT1-4, respectively, which display distinct cellular and regional expression patterns and ionic requirements (Borden, 1996; Conti et al., 2004). **GAT-1** is the most abundant high-affinity neuronal GABA transporter found predominantly on GABAergic neurons and some astrocytes (Uhl, 1992). Beside its expression in the CNS, GAT-1 was described in the testis and sperm of rodents (Hu et al., 2000; Ma et al., 2000). Three other closely related transporters show specific pattern of distribution: **GAT-4** was detected only in the brain, while **GAT-2** and **GAT-3** are present in both the CNS (brain and retina) as well as a number of non-neuronal tissues such as liver and kidney (Liu et al., 1993; Borden, 1996; Jursky and Nelson, 1996, 1999).

Physiological and biochemical studies have demonstrated that GABA can be released by a reversal of GATs, a mechanism predominately utilized by the growing cones of neuronal precursors during synaptogenesis (Pin and Bockaert, 1989; Taylor et al., 1990; Taylor and Gordon-Weeks, 1991; Attwell et al., 1993; Levi and Raiteri, 1993; Wu et al., 2007).

1.1.3. *GABA receptors as mediators of GABA action*

GABA exerts its action by binding to the two main types of receptors: ionotropic, $GABA_A$ and $GABA_C$, which mediate fast inhibitory neurotrasmission, and metabotropic $GABA_B$, which is coupled to GTP-binding proteins and mediates slow inhibitory neurotransmission. In the adult brain, $GABA_A$ and $GABA_B$ receptors are abundantly expressed mainly in neurons but also in glia.

1.1.3.1. Ionotropic GABA ($GABA_A$ and $GABA_C$) receptors

Most of the physiological actions of GABA are mediated by **$GABA_A$** receptors. Functional $GABA_A$ receptors are heterooligomeric assemblies of distinct subunits that form a membrane channel permeable to chloride, and to a lesser extent to bicarbonate anions (Kaila, 1994). In mammals 19 receptor subunits have been identified that are grouped into 4 subgroups α (α_{1-6}), β (β_{1-3}), γ (γ_{1-3} and the γ substitutes ∂, ε, π ,θ). Additional forms derive from alternatively spliced variants (Barnard et al., 1993; Simon et al., 2004). All subunits are similar in size, containing roughly 450-550 amino acids and are strongly conserved among species. A high percentage of sequence identity (70-80%) is found between subunit isoforms and, to a lesser extent (30%) between subunit families (***Fig.4A***). The common elements of the subunit structure include a large N-terminal hydrophilic domain (containing a signal peptide, a cysteine bridge and glycosylation sites) exposed to the synaptic cleft and four hydrophobic segments named M1 to M4, each composed of approximately 20 amino acids forming four putative membrane-spanning segments (***Fig.4B***). In theory, a large number of various combinations could assemble from the numerous subunits and their splice variants. However, studies reveal that only a limited number of these combinations can be found on the cell surface (Turner, 1993; Fritschy and Mohler, 1995; Sieghart and Sperk, 2002). Most, in not all $GABA_A$ receptors form heteropentameric assemblies containing two α, two β and one γ (or ∂, ε, π, θ) subunit (***Fig.4C,D***). In mammalian central and peripheral nervous system, activation of $GABA_A$ generates, in general membrane hyperpolarization and a reduction of action potential firing that leads to both phasic inhibitory postsynaptic currents (IPSCs) and inhibitory tonic currents (Kaneda et al., 1995; Nusser and Mody, 2002). The GABA binding sites are located at the α and β subunit interfaces (see ***Fig.4C***), whereas receptor specific agonists and antagonists can bind at other locations (Baumann et al.,

2001). Due to the diversity in subunit composition, $GABA_A$ receptor neurotransmission is characterized by high variability of synaptic responses. The kinetics and pharmacological diversity of $GABA_A$ receptor response is particularly evident during development (Schousboe and Redburn, 1995; Costa, 1998; Cherubini and Conti, 2001).

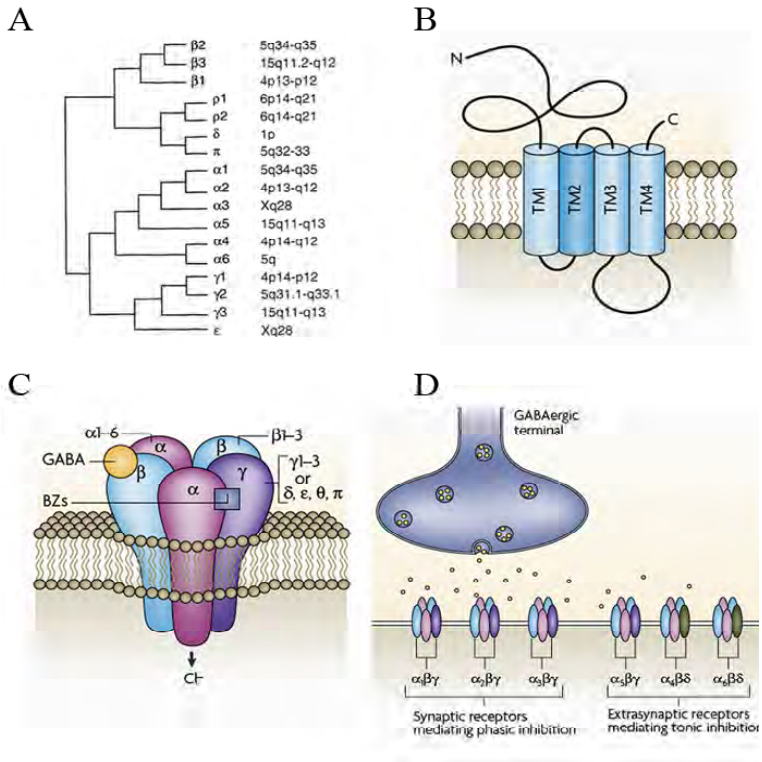


Fig.4. $GABA_A$receptor structure and localization. **A**. The human $GABA_AR$ gene family. The dendogram indicates the homologies between deduced amino-acid sequences of subunits. **B**. $GABA_AR$ subunits consist of four hydrophobic transmembrane domains (TM1–4). The large extracellular amino terminus is the site of GABA binding, and also contains binding sites for psychoactive drugs such as benzodiazepines (BZs) **C.** Five subunits from seven subunit subfamilies (α, β, γ, δ, ε, θ and π) assemble to form a heteropentameric Cl^--permeable channel. Binding of the neurotransmitter GABA occurs at the interface between the α and β subunits and triggers the opening of the channel, allowing the rapid influx of Cl^- into the cell. **D**. $GABA_ARs$ composed of α(1–3) subunits together with β and γ subunits are thought to be primarily synaptically localized, whereas α(4-6)βγ/δ receptors are located largely at extrasynaptic sites Modified in part *from Jacob et al., 2008.*

$GABA_C$ receptors are ligand–gated Cl^- channels assembled as homo- or heteropentamers from three distinct ρ (ρ1-3) subunits (Bormann, 2000; Zhang et al., 2001). They are found primarily in the vertebrate retina and at restricted sites in the spinal cord and other parts of the CNS (Johnston, 1996; Lukasiewicz, 1996).

1.1.3.2. $GABA_B$ receptor

Metabotropic $GABA_B$ receptors were first described in 1981 as a bicuculline-insensitive, baclofen-sensitive GABA receptor type widely expressed in the mammalian central nervous system (Hill and Bowery, 1981). Functional $GABA_B$

receptors are heterodimers of $GABA_{BR1}$ and $GABA_{BR2}$ subunits (***Fig.5***; Bowery and Brown, 1997; Kaupmann et al., 1997). Heterodimerization of the two subunits is absolutely critical for efficient receptor trafficking to the cell membrane, formation of ligand-binding sites, and coupling to downstream effectors (Jones et al., 1998). Deletion of either of the subunits results in a complete loss of $GABA_B$ receptor functions (Couve et al., 2000,2004; Bowery, 2006; Mombereau et al., 2004)

The cloning of $GABA_{BR1}$ revealed the existence of several additional alternatively spliced forms in rodents and human (Couve et al., 1998). The first two initially discovered $GABA_B$ receptor isoforms were termed $GABA_{BR1a}$ and $GABA_{BR1b}$ with molecular weights of 130 and 100kDa, respectively. These two isoforms differ by the insertion of a pair of tandem extracellular "Sushi" domains at the N-terminus of $GABA_{BR1}$, which have been previously shown to be involved in protein-protein interactions (Hawrot et al., 1998). Whereas the specific function of these different isoforms is unclear, they may provide a mechanism for differential targeting of $GABA_B$ receptor within the cell or the coupling of these receptors to different effector systems (Vigot et al., 2006).

With respect to native receptors in the brain, $GABA_B$ receptors can be separated into a postsynaptic population that controls postsynaptic excitability and a presynaptic population that regulates neurotransmitter release. Postsynaptic $GABA_B$ receptors induce slow inhibitory postsynaptic currents (sIPSP) by gating (or activating of inwardly rectifying potassium channels (GIRK or Kir3-type K+ channels), which evokes hyperpolarizes action of the membrane and shunting of excitatory currents (Ong and Kerr, 2000). Presynaptic $GABA_B$ receptors can function as autoreceptors and control GABA release or heteroreceptors that inhibit the release of other neurotransmitters, especially glutamate. These receptors mediate their presynaptic effects mostly by inhibition of high-voltage activated Ca^{2+} channels of N or P/Q-type

or through direct modulation of synaptic vesicle priming (Mintz and Bean, 1993; Poncer et al., 1997; Sakaba and Neher, 2003). Furthermore, activation of $GABA_B$ receptors inhibits cAMP production (Hashimoto and Kuriyama, 1997; Simonds, 1999) predominately through G proteins of the $G_{i/o}$ family (***Fig.5;*** Bowery et al., 2000; Odagaki and Koyama, 2001; Bettler et al., 2002).

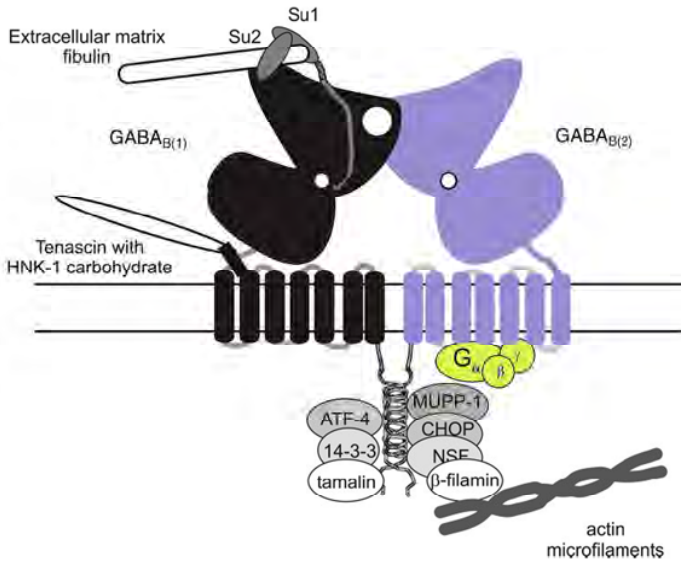


Fig.5. Schematic representation of $GABA_B$ receptor structure. Through the activation of Gi / Go -type G proteins $GABA_B$ receptors couple to pre- and postsynaptic effector systems. A number of proteins were found to interact with the cytoplasmic tails of $GABA_B$ subunits. Among the interacting proteins are leucine-zipper transcription factors (CREB2/ATF-4, CHOP), suggesting that $GABA_B$ receptors mediate long-term metabolic changes involving protein synthesis. Furthermore, a number of scaffolding and adaptor proteins such as 14–3-3, *N*-ethylmaleimide-sensitive factor (NSF), tamalin, and MUPP-1 were found to interact with the coiled-coil domains of either $GABA_BR1$ and/or $GABA_BR2$. It is proposed that these proteins regulate receptor dimerization, intracellular trafficking, and synaptic localization. -Filamin, an actin binding protein, is supposed to tether the $GABA_B$ heterodimer to the cytoskeleton via interaction with the $GABA_BR2$ subunit. The extracellular sushi repeats (Su1 and Su2) of $GABA_BR1a$ are proposed to interact with the extracellular matrix protein fibulin. HNK-1, a carbohydrate carried by extracellular matrix proteins such as tenascin, binds to the extracellular domains of both $GABA_B$ receptor subunits. Modified from *Bettler et al., 2004.*

1.2. Mechanism of Depolarizing/Excitatory actions of GABA

The inhibitory role of GABA on membrane excitation in the adult brain is reversed in the embryonic nervous system, where GABA exerts mainly an excitatory role (Owens and Kriegstein, 2002; Ben-Ari, 2007). Numerous studies in rodents revealed that activation of $GABA_A$ receptors, in immature neurons results in efflux, rather then influx of chloride ions and subsequently inward electric current producing membrane depolarization (Owens and Kriegstein, 2002; Ben-Ari et al., 2007) generating action

potentials. Activation of $GABA_A$ receptors also increases intracellular calcium concentration ($[Ca^{2+}]_i$) due to the activation of voltage-gated calcium channels (***Fig.6***; Obrietan and van den Pol, 1995), attenuates the voltage–dependent magnesium block of NMDA channels (***Fig.6***; Leinekugel et al., 1997) and interferes with ionotropic glutamatergic transmission (Gao et al., 1998).

1.2.1. Developmental expression of KCC2

Members of cation chloride co-transporter family regulate ion homeostasis through inward or outward directed flux of ions, which depends on electrochemical gradients. The gradient of potassium (K^+) is regulated by the K+/Cl- co-transporters (KCC), which extrude Cl^- out of the cell whereas the sodium (Na^+) gradient is regulated by $Na^+/K^+/Cl^-$ co-transporters (NKCC) to accumulate Cl^- within the cell. Until now, four K^+/Cl^- co-transporters (KCC1-4) and two $Na^+/K^+/Cl^-$ co-transporters (NKCC1-2) have been described: **NKCC1** and **KCC1/3/4** are generally expressed in all tissues, **NKCC2** is restricted to kidney, while **KCC2** is only found in neuronal tissue. (Delpire and Mount, 2002; Payne et al., 2003).

The developmental shift of GABA effect on the cells from excitatory to inhibitory is thought to be caused by a decrease in the intracellular Cl^- concentration in developing neurons (***Fig.6***; Rivera et al., 1999; Payne et al., 1996,1997). This is supported by numerous studies that have demonstrated a correlation between the GABA reversal potential and expression level (Lu et al., 1999; Rivera et al., 1999; Coull et al., 2003; Shibata et al., 2004), membrane localization (Balakrishnan et al., 2003), phosphorylation (Kelsch et al., 2001), and/or activity of KCC2 (Woodin et al., 2003). Loss-of-function studies using antisense KCC2 RNA (Rivera et al., 1999), targeted gene knockdown (Hubner et al., 2001; Woo et al., 2002; Zhu and Delpire, 2005) or

over-expressing KCC2 in vitro studies (Lee et al., 2005) have shown that KCC2 is necessary and sufficient for establishing and maintaining a low $[Cl^-]i$.

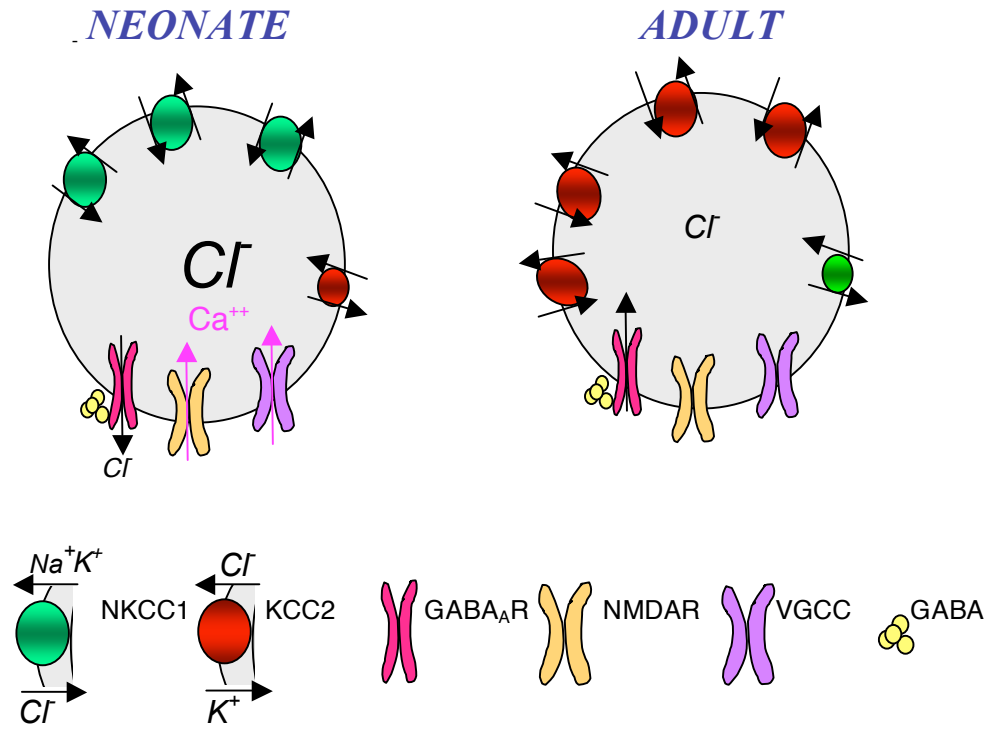


Fig.6. Schematic presentation demonstrating early expression of NKCC1 and late expression of KCC2 determines developmental changes in intracellular Cl^- concentration and in the GABA-induced membrane depolarization/hyperpolarization. Note that NKCC1 expression predominates in immature neurons, in which the intracellular concentration of chloride ($[Cl^-]i$) is relatively high and sufficient to cause the GABA reversal potential to be either below or above the resting membrane potential and the threshold for action potential generation .

1.3. GABA signaling during development

In addition to being the principal inhibitory neurotransmitter in the adult nervous system, GABA is thought to play a signaling role during development acting in a paracrine/autocrine fashion (Manent et al., 2005; Manent and Represa, 2007) and reg ulating 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;Varju et al., 2000; Owens and Kriegstein, 2002; Ben-Ari, 2002).

1.3.1. Summary of nervous system development

During early development, the layer of **germinal neuroepithelium** consists of a monolayer of rapidly dividing neural stem cells. During the cell cycle, their nuclei undergo "interkinetic nuclear migration" migrating to the pial side of the neural tube during S-phase and back to the luminal side during M-phase (Gilbert, 2000). Extensive proliferation within the germinal neuroepithelium during early developmental stages leads to the generation of two different cell populations forming the **ventricular zone (VZ; *Fig.7A*)**: radial glia neural stem cells through symmetric, and neuroblasts through asymmetric cell division. The neural stem cells can divide asymmetrically to generate both neuronal progenitors and radial glia neural stem cells. At mid-neurogenesis a secondary proliferative zone is formed, the **subventricular zone (SVZ)** generating neurons and astroglia. After further thickening of the cerebral wall, neuronal progenitors migrate along radial glia fibres and settle above the SVZ, building the **mantle zone (MZ)** and below the newly forming **cortical plate (CP; *Fig.7A*)**. Early-born neurons settle in deeper layers and late born cells remain closer to the pial surface following an inside-out gradient of migration (Rakic, 1990). Cells stop migrating at CP/MZ interphase as they adopt their final position in the CP. There are two main neuronal types of the mammalian cerebral cortex: excitatory pyramidal cells and inhibitory non-pyramidal cells and they are generated in distinct proliferative zones. Pyramidal neurons arise from the germinal ventricular zone (VZ) and migrate along the radial glia processes of to take up their positions in the developing cortex (Rakic, 1990), while a diverse group of mostly γ-aminobutyric acid (GABA)-ergic interneurons originate predominantly in the **medial ganglionic eminence** (**MGE**) of

the ventral telencephalon and navigate along a multiple **tangential** migratory routes through the **intermediate zone** (**IZ**) to reach the cortex (***Fig.7B***, De Carlos *et al.*, 1996; Anderson *et al.*, 1997a,b, 2001)

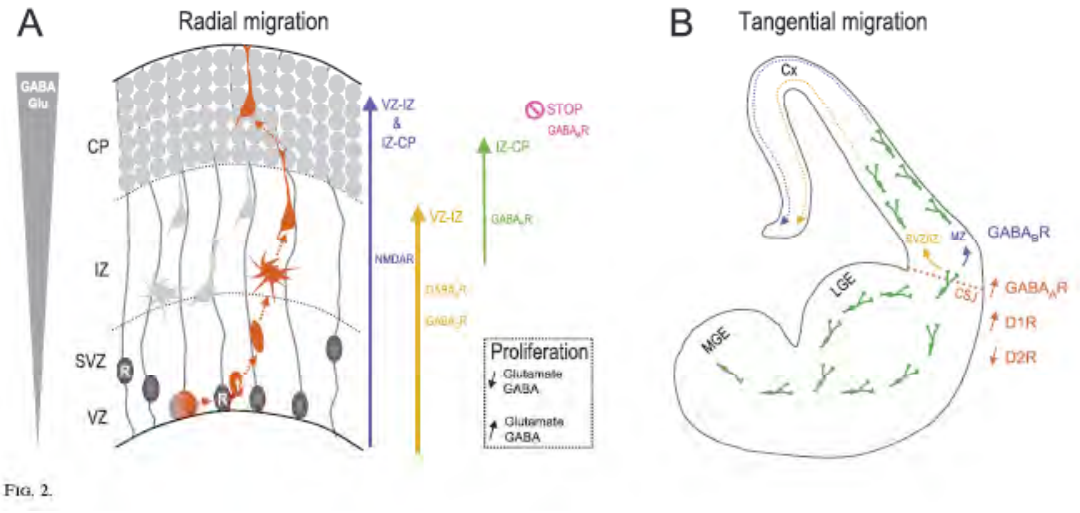


Fig.7. GABA and other neurotransmitters regulates neuronal migration in the developing telencephalon. (A) Schematic diagram showing the cortical wall of an E13.5 mouse brain.This diagram illustrates the functions undertaken by neurotransmitters and their cognate receptors in the regulation of progenitor proliferation and migration of newborn projection neurons within the cortical wall. γ-Aminobutyric acid (GABA) and glutamate (Glu) are distributed along an apical to basal concentration gradient. By activating GABAAR or AMPA / kainate receptors, GABA and Glu regulate cell proliferation in opposite ways in ventricular zone (VZ) and subventricular zone (SVZ) progenitors (LoTurco et al., 1995; Haydar et al., 2000). While Glu stimulates radial migration along the entire cortical wall (Behar et al., 1999), GABA triggers the ionotropic receptors $GABA_AR$, $GABA_CR$, which in turn promote the migration of projection neurons from the VZ to the intermediate zone (IZ). Importantly, activation of the metabotropic GABABR is specifically required for the transition of cortical projection neurons from the IZ to the caudate putamen (CP; Behar et al., 2000). Finally, activation of $GABA_AR$ also acts as a stop signal for projection neurons in the CP (Behar et al., 1998). B) GABAergic interneurons (green cells) are mainly generated in the medial ganglionic eminence (MGE) and follow distinct tangential migratory routes to integrate into the cerebral cortex (Cx). After having avoided the presumptive striatum, interneurons cross the corticostriatal junction (CSJ), which corresponds to the interface between the subpallium and the pallium, and enter the cortical anlage. These cells then follow distinct routes within the cortical wall (the VZ / SVZ and the MZ at early stages of corticogenesis). GABA promotes the crossing of the CSJ by triggering interneuron GABAAR activation (Cuzon et al., 2006), and influences the choice of cortical migratory route by acting on the metabotropic GABABR (Lopez-Bendito et al., 2003). LGE, lateral ganglionic eminence; NMDAR, N-methyl-d-aspartate receptor. *Heng et al., 2007.*

1.3.2. Molecular components of the GABA signaling pathway are expressed during neuronal development in a strict spatio-temporal manner

Components of GABA signaling system, GABA and GABA-synthesizing enzymes, GABA receptors and GABA transporters appear transiently during development (Buznikov et al. 1996; Zecevic and Milosevic 1997; Barker et al. 1998; Verney 2003; Lujan et al. 2005). The embryonic and adult GAD mRNAs and protein forms are expressed in the embryonic and postnatal development of the nervous system in a

highly specific temporal fashion (Behar et al., 1994; Ma et al, 1994; Szabo et al., 1994; Katarova et al., 2000, reviewed in Varju et al., 2000, 2001). 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). Concomitant with neuronal maturation and synaptic formation embryonic forms are gradually replaced by the adult GAD forms (Szabo et al., 1994; Somogyi et 1995; Varju et al., 2001). These results led to the speculation that the embryonic GAD proteins may be involved in the synthesis of morphogenic GABA while adult GADs provide GABA for synaptic and metabolic action in the mature GABAergic neurons (Szabo et al., 1994; Varju et al., 2000).

Studies of neurons in culture suggest an earlier presence of $GABA_A$ receptors and currents than other transmitters, including glutamate and glycine (Lujan et al., 2005; Owens and Kriegstein, 2002; Ben-Ari et al., 2008). Several types of $GABA_A$ receptor subunits have been detected and proved functional in rat embryo as early as E10-E11 by in situ hibridization, immunocytochemistry and pharmacological studies (Laurie et al. 1991, 1993, Bettler, Haydar et al., 2000, Behar et al., 1993). Whole-cell recordings made from embryonic and early postnatal cells have shown that early expressed $GABA_A$ receptors change kinetic properties during development (Owens and Kreigstein, 1999). Differences in receptor properties might be due to the differences in subunit composition, intracellular calcium concentrations, or degree of receptor phosphorulation and association with cytoskeletal anchoring proteins (Lujan et al., 2005; Owens and Kriegstein, 2002; Ben-Ari et al., 2007).

Even though functional $GABA_B$ receptor requires heterodimerization of $GABA_BR1$ and $GABA_BR2$ subunits, they display different expression patterns during embryonic development, indicating they are regulated independently. As revealed by in situ hybridization, $GABA_BR1$ receptor subunit mRNA is intensively expressed by E11,

and from E12 on is detected in the rat hippocampus, cerebral cortex, intermediate and posterial neuroepithelium (Kim et al., 2003; Martin et al., 2004; Li et al., 2004). In contrast, a weak expression of $GABA_B$ R2 mRNA in the same brain areas could be detected only from later developmental stages (Li et al., 2004).

Both membrane (GAT-1, GAT-3 and GAT4) and vesicular transporter (VGAT) mRNAs have been detected at early stages of mouse forebrain development however their function in differentiating neurons has not been studied in detail yet (Jursky et al., 1996; Oh et al., 2005; Fukui et al., 2008).

1.3.3. GABA as a modulator of neurogenesis

1.3.4.1. GABA modulate proliferation and survival of neuronal progenitor cells

Emerging evidence indicates that GABA exerts modulatory, often contrasting effect on cell proliferation depending on the cell and GABA receptor type (**Fig.7A;** Owens and Kriegstein, 2002; Lujan et al., 2005; Ben-Ari et al., 2007). Estimation of [^{3}H]-thymidine or bromodeoxyuridine (BrdU) incorporation in cells derived from the E16-E19 cortex has revealed that GABA, activating $GABA_A$ receptors inhibited DNA synthesis in proliferating cells. This cell cycle arrest was associated with depolarization and Ca^{2+} entry as high potassium and glutamate treatments had the same effects. (LoTurco et al., 1995; Owens et al., 1996). Conversely, in rat immature cerebellar granule cells GABA promoted proliferation by indirect activation of the mitogen-activated protein kinase (MAPK) cascade (Fiszman et al., 1999). A marked difference in the rate of proliferation in response to GABA between cells isolated from the different germinal layers of embryonic mouse brain was also demonstrated. In 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). Both effects seem to be mediated by $GABA_A$ receptor since treatment with $GABA_A$-receptor agonists mimics this action, whereas antagonists completely abolish it.

1.3.4.2. GABA regulates neuronal migration

Once neuroblasts are generated in the germinal layers, they undergo terminal mitosis and migrate to their final destination in the cerebral tissue. In cultured rat brain slices, activation of $GABA_B$- and $GABA_C$ receptors stimulate the migration from the VZ and IZ (intermediate zone), respectively, while activation of $GABA_A$ receptors acts as a „stop" signal for neurons that reached the CP (***Fig.7A:*** Behar et al., 1998). Blocking of $GABA_A$ receptors resulted only in a delay of cell movements, but not a complete arrest of migration (Behar et al., 2000). Furthermore, in the developing rat cortex, GABA is transiently expressed in the CP, near target destinations for migrating neurons (Lauder et al., 1986; Barker et al., 1998). In vitro, femtomolar GABA or baclofen stimulated gradient-dependent or direct neuronal migration from VZ, while micromolar levels induced random migration of neurons isolated from CP. Migration was completely blocked by saclofen, $GABA_B$ receptor antagonist (Kerr and Ong, 1992; Behar, 2001) indicating that GABA act as chemoattractants for neurons during rat cortical histogenesis via mechanisms involving $GABA_B$ receptor (Behar, 2001).

Recently, several studies demonstrated modulatory role of GABA during tangential migration supporting the notion of involvement of $GABA_B$ receptors in neuronal migration (***Fig.7B***; Heng et al., 2007).

1.3.4.3. GABA as a modulator of neuronal differentiation

In addition to proliferation and migration, a number of studies demonstrated that GABA-mediated signaling is important during synaptogenesis and neuronal differentiation (Redburn and Schouusboe, 1987; Lujan et al., 2005). In pioneer studies by Wolff and colleagues demonstrated that continuous application of GABA to the rat superior cervical ganglion *in vivo* stimulated dendritic growth and sprouting (Wolf et

al., 1978, 1987). In cultured embryonic hippocampal and neocortical neurons, $GABA_A$ receptor activation promoted neurite outgrowth and maturation of GABA interneurons (Barbin et al., 1993; Marty et al., 1996; Maric et al., 2001; reviewed in Lujan et al., 2005).

1.4. GABA signaling outside the nervous system

As mention above, GABA as well as different molecular components of GABA signaling have been detected in many tissues and cell types throughout the body including the pancreas, kidney, lung, liver, adrenal gland, platelets and erythrocytes, endocrine cells of the pineal and pituitary, oocytes, male and female reproductive organs, chondrocytes of the growth plate, intestinal epithelial cells and keratinocytes (Lancaster et al., 1975; Kataoka et al., 1984; Erdo and Amenta, 1986a, 1986b; Garry et al., 1986,1987,1988; Erdö et al., 1989; Gilon and Remacle, 1989; Erdö and Wolff, 1990; Parducz et al., 1992; Tillakaratne et al., 1992; Gilon et al., 1997; Calver et al., 2000; Erlitzki et al., 2000; Jin et al., 2005; Tamayama et al., 2005; Xiang et al., 2007; Mizuta et al., 2008a, 2008b).

In contrast to CNS, the role of GABA in these tissues is not so well defined. However, there is plenty of evidence of diverse GABA functions in non-neuronal tissues such as modulation of proliferation in pancreatic islet cell and tibial growth plate, regulation of hepatic regenerative activity, motility and agglutination of spermatozoa, stimulation of testosterone production, regulation of uterotubal and gut motility, and control of hormone release in ovary, adrenal medulla, pancreas and gastrointestinal tract and differentiation of epithelial cells of the jejunum (Kataoka et al., 1984; Louzan et al., 1986; Racagni et al.,, 1986; Gilon et al., 1991; Tillakaratne et al., 1995; Tamayama et al., 2001; Wang et al., 2005; Ligon et al., 2007).

Furthermore, GADs were shown to be expressed in non-neural tissues of the mouse embryo such as the tail bud mesenchyme, the pharyngeal pouches and arches, the ectodermal placods, the apical ectodermal ridge, mesenchyme and ectoderm of the limb buds, olfactory epithelium, heart, endothelial cells of blood vessels and lens fibers indicating that GABA might have a role also in the development of a variety of tissues (Li et al., 1995; 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 of the palate (Maddox and Condie, 2001; Hagiwara et al., 2003). In addition, cleft palate phenotype was observed in the offspring of mice treated during pregnancy with $GABA_A$ antagonist (and agonists) or GAD inhibitor (Ding et al., 2004).

1.4.1. GABA influences cancer cell proliferation and metastasis

Recently, several studies demonstrated potential role of GABA in cancer cell proliferation (Watanabe et al., 2006). Increased GAD activity and GABA content have been reported in colon cancer (Kleinrok et al., 1998; Maemura et al., 2003), breast cancer (Opolski et al., 2000), gastric cancer (Matuszek et al., 2001), and glioma (Bianchi et al., 2004). Increased expression of different GABA receptor subunits have been detected in neuroblastomas (Roberts et al., 2004), liver cancer (Biju et al., 2002), and breast cancer (Jiang et al., 2002). Increased GABA and GAD expression was found in prostate cancer patients with metastasis. In the latter, authors demonstrated that GABA promoted cancer cell invasion via $GABA_B$ receptor pathway, and an increase in MMP (matrix metalloproteinase) activity was an underlying mechanism of action (Azuma et al., 2003).

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.

2. EMBRYONIC STEM (ES) CELLS

2.1. Origin of mouse ES cells

Stem cells are a unique for their ability for substantial self-renewal and the potential to differentiate into various cell types. Stem cells that are capable of differentiation into cells of all three germ layers – ectoderm, mesoderm and endoderm are defined as **pluripotent stem cells**. Three types of pluripotent stem cells have been described so far; i) embryonic germ (EG) cells of the gonads of a postimplantation embryo, ii) embryonic carcinoma (EC) cells, isolated from embryo-derived teratocarcinomas, and iii) embryonic stem cells, derived directly from the blastocyst-stage embryo without use of immortalizing or transforming agents (Gottlieb, 2002).

Embryonic stem (ES) cell lines were originally isolated from in vitro outgrowths of inner cell mass (ICM) of mouse blastocysts (Evans and Kaufman 1981; Martin 1981). To date, various ES cell lines have been generated from other species including humans (Thomson *et al.*, 1998). Although a layer of mitotically inactivated mouse fetal fibroblasts, also called feeder cells, was necessary for the initial isolation of ES cells (Martin, 1981), it was subsequently shown that for maintenance of an established culture in its undifferentiated state a soluble factor identified as leukemia inhibitory factor (LIF) was required, which then became the standard ingredient of ES cell medium.

In cultured ES cells, STAT3 activation by the cytokine LIF is required to sustain the self-renewal (Niwa et al., 1998; Matsuda et al., 1999), however it is not required for normal mouse development (Stewart et al., 1992).

More recently, other factors in addition to LIF were shown to contribute to maintenance of ES cell self-renewal. These include members of BMP (Ying et al, 2003) and the Wnt families (Sato et al., 2004).

2.2. Properties of undifferentiated ES cells

ES cells multiply by symmetrical cell divisions, which are not restricted by contact inhibition (Fuji-Yamamoto et al., 2005). It has been demonstrated that their cell cycle is characterized by a virtual lack of the G1 and G2 gap phases, thus approximately 50% of the ES cells in culture sojourn in the S phase at any given time-point. (Jirmanova et al., 2002). Furthermore, mouse ES cells can proliferate for years in an undifferentiated state while maintaining the potential to give rise to the majority of cell types found throughout the whole body. Intact embryos do not normally maintain unlimited proliferation of undifferentiated cells meaning that ES cells could be considered *in vitro* culturing artifacts (Martin, 1981; Martin and Evans, 1980). However, these cells provide a stable model of embryonic growth and development and a tool for investigation specific signaling systems.

Identification of cell surface and molecular markers has proven critical to define the molecular basis of stem cell identity. It is now well established that mouse ES cells express specific cell surface antigens (SSEA-1) and membrane-bound receptors (gp130) and possess enzyme activity for alkaline phosphatase (**ALP**) and telomerase (**Tert**) (Wobus et al., 2005; Amstrong et al., 2000). To date, there are only a few transcription factors whose appropriate level of expression is necessary to maintain pluripotency of cell population in pre- and early postimplantation embryos. **Oct4** is developmentally regulated cell-restricted transcription factor. In the early embryo it is abudantly expressed in the inner cell mass, and down-regulated in the trophoectoderm (***Fig.8***; Pesce and Scholer, 2001). Oct3/4-deficient embryos form a blastocyst, but the internal cells of the prospective ICM are unable to produce extraembryonic endoderm or other differentiated derivates and rather become diverted into the trophoectoderm lineage (Nichols et al., 1998). The recently identified homeodomain protein **Nanog** is another key regulator of ES cell pluripotency (Mitsui et al., 2003; Hart et al., 2004). In

preimplantation embryos, its expression is restricted to and required in epiblast cells from which ES cells can be derived (***Fig.8***). Other molecular markers potentially defining pluripotency include Rex-1, **Sox-2**, Genesis, GBX2, UTF1, Pem and L17. All of these are expressed in the ICM of the blastocyst and are downregulated upon differentiation, but can be found in other cell types as well (Niwa et al., 1998, 2000, 2001, 2007). The ES cell property of self-renewal therefore depends on the stoichiometric balance among various signaling molecules, and an imbalance in any one can cause ES cell identity to be lost (***Fig.7***).

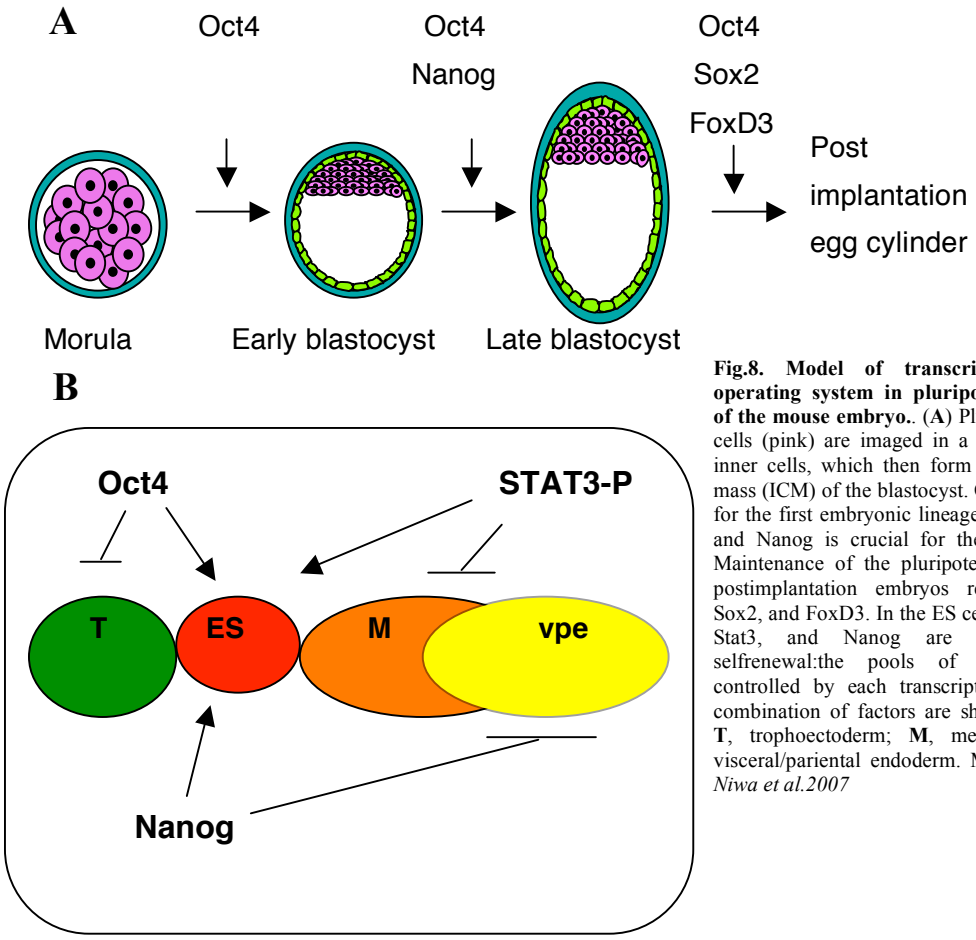


Fig.8. Model of transcription factor operating system in pluripotent lineages of the mouse embryo.. (**A**) Pluripotent stem cells (pink) are imaged in a morula as the inner cells, which then form the inner cell mass (ICM) of the blastocyst. Oct4 is crucial for the first embryonic lineage specification, and Nanog is crucial for the second. **(B)** Maintenance of the pluripotent epiblast of postimplantation embryos requires Oct4, Sox2, and FoxD3. In the ES cell Oct4, Sox2, Stat3, and Nanog are essential for selfrenewal:the pools of target genes controlled by each transcription factor or combination of factors are shown in color. **T**, trophoectoderm; **M**, mesoderm; **vpe**, visceral/pariental endoderm. Modified from *Niwa et al.2007*

3. THE OCULAR LENS

3.1. An overview of mouse lens development

The lens is a highly specialized, transparent structure inside the eye that, along with the cornea, helps to refract light so as to be focused on the retina. Moreover, by changing shape, lens can 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.

The establishment of the unique architecture of the lens in the embryo is thought to depend on a series of inductive interactions that initiate its differentiation (reviewed in McAvoy, 1999). The current view is that lens induction begins during mid to late gastrulation, when involuting endoderm and mesoderm interacts with the adjacent prospective head ectoderm to give a lens-forming bias. The activation of the head ectoderm's latent lens-forming ability and the positioning of the lens in relation to the retina is accomplished by the **optic vesicle** (***Fig.9***). The optic vesicle bulges out of the diencephalon, and induces the formation of the **lens placode** (***Fig.9***) from the overlying head ectoderm. Subsequent invagination of the placode forms the **lens pit**, which later closes to form the **lens vesicle** (***Fig.9***). Cells in the posterior segment of the lens vesicle, next to the optic cup, elongate to form the **primary fibers**, whereas cells in the anterior segment of the vesicle become the lens epithelial cells. These divergent fates of embryonic lens cells give the lens its distinctive polarity. From this stage onwards, the lens grows by continued proliferation and differentiation of epithelial cells into fiber cells. Proliferation initially occurs throughout the lens epithelial compartment but at later developmental stages becomes progressively restricted to a band of cells above the equator, known as **germinative zone** (***Fig.9***).

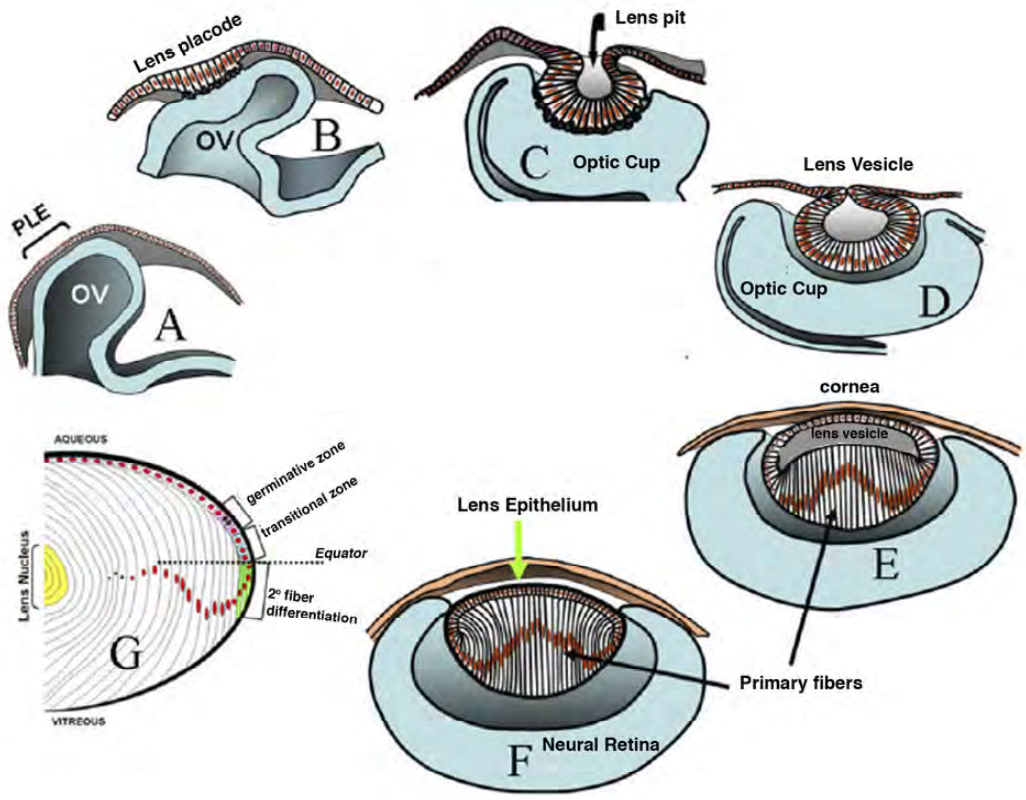


Fig. 9. Schematic view of a developing mouse eye. A. Morphological development of the lens begins as the optic vesicle (OV) approaches the presumptive lens ectoderm (PLE). **B.** PLE thickens to form the lens placode (E9.5). **C.** The lens placode invaginates forming the lens pit and the OV evaginates forming the optic cup (E10.5). **D.** The lens pit deepens and close to form 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 aterior 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 primary fiber cells. Modified by *Robinson, 2006*

The lens epithelial cells are sequestered into four regions known as:

a) a central zone (cz) – a broad polar cap of the LE covering 80% of anterior surface of the lens arrested in the G0 stage of the cell cycle (Harding et al., 1971)

b) pregerminative zone (pgz) – comprising 5% of LE and "daughter cells" add to the population of the lens epithelium as the anterior surface of the lens increases in size during aging (Rafferty and Rafferty, 1981);

c) germinative zone (gz) – stem cells, which undergo mitotic division, then migrate towards the equator where they elongate adding to already existing differentiated fibers (***Fig.9***) and

d) transitional zone (tz)– nascent fibers having already begun the process of elongation (comprises approximately 5% of LE ; ***Fig.9***) towards both lens poles.

The ocular lens grows throughout life in both diameter and cell number as equatorial epithelial cells elongate, lose all their intracellular organelles, synthesize crystallins- the major lens cytoplasmic proteins (α, β, γ in mammalians), and an integral membrane protein known as the major intrinsic protein (MIP) (Alcala et al., 1975; Bloemendal et al., 1989,2004; Bassnett et al., 2002, 2008; Bhat, 2003)

3.2. Multiple factors controlling lens development

Distinct transcriptional regulators and growth factors are implicated in the initiation and maintenance of lens structure (***Fig.10***). A number of recent transgenic studies provided evidence that retinoic acid and members of BMP and FGF family genes play important roles as inductive signals in the lens at early stages. For example, expression of BMP genes are essential for the proper formation of lens placode (Furuta and Hogan, 1998; Wawersik et al., 1999) while the fibroblast growth factor (FGF), appears to be a key initiator during later developmental stages- lens fiber differentiation and maintenance (***Fig.10***; Robinson et al., 1995a, 1995b; Stolen and Griep, 2000). Other factors, such as transforming growth factor (TGF), platelet-derived growth factor (PDGF-A), insulin-like growth factor (IGF-1), epidermal growth factor (EGF) and hepatocyte growth factor (HGF) are also involved in lens cell proliferation and differentiation (reviewed in Lovicu and McAvoy, 2005).

A number of transcription factors have recently been identified as regulators of eye and lens development, including homeobox genes Pax6, Six3, Prox-1 and Sox2 (***Fig.10***: Ogino and Yasuda, 2000). Finally, most transcription factors involved in lens development have additional roles, in regulating other aspects of eye morphogenesis (Jean et al., 1998).

Although eye development is one of the most extensively studied processes in the embryo, the identity and specific roles of many regulatory factors controlling lens differentiation are still obscure.

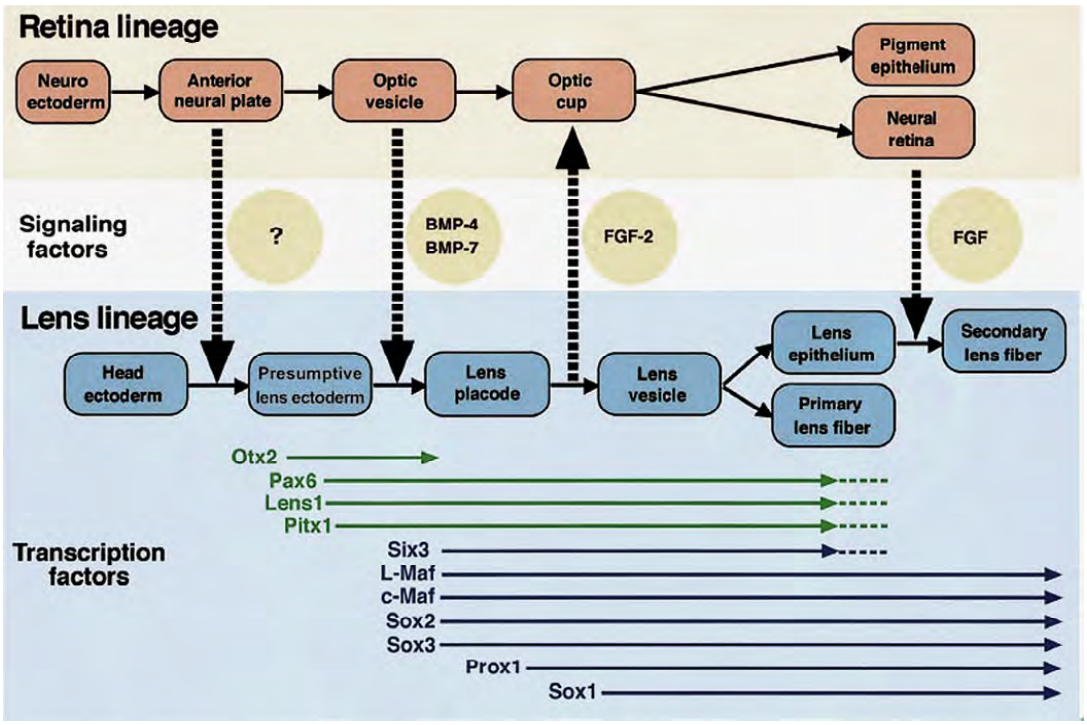


Fig.10..Diagram showing the link between inductive interactions, expression of transcription factors during lens development Developmental stages (at the top) and major inductive events between the retina- and lens-forming tissues (bold, dotted arrows) are illustrated according to the currently favored 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. Modified from Ref.: *Ogino and Yasuda, 2000.*

IV OBJECTIVES/AIMS

The major objective of this thesis was to characterize the GABA signaling components and functionality during differentiation using two different model systems: mouse embryonic stem (ES) cells and the ocular lens. Using this two model systems will allow us uncovering the role of GABA in developmental processes such as proliferation, differentiation and migration and the general mechanism of action, which could also apply to the nervous system.

The specific aims followed in the course of this work could be summarized as follows:

(1) Identification and expression characterization of the GABA signaling components in undifferentiated ES cells and in the course of retinoic acid-induced neuronal differentiation: GAD forms and the two transcriptional regulators of GAD - Dlx2 & 5, $GABA_A$ and $GABA_B$ receptor subunits, vesicular and membrane GABA transporters.

(2) To probe the functionality of the GABA signalling in the ES cells by studying the effect of $GABA_A$ and $GABA_B$ receptor activation on the intracellular calcium levels using digital calcium imaging and confocal microscopy.

(3) To determine the effect of GABA and GABA receptor ligands on ES cell proliferation and differentiation.

(4) Characterization of the spatio-temporal expression pattern of GABA signalling components in the developing mouse lens: GAD forms, Dlx2&5, GABA, $GABA_A$ and $GABA_B$ receptor subunits, vesicular and membrane GABA transporters;

(5) Using calcium-imaging to test the functionality of the GABA signalling in the lens and in parallel, in primary lens epithelial cell (LEC) cultures prepared from mouse lenses.

V METHODS

1. ANIMAL HANDLING

Wild type C57Bl6 and FVB/Ant mice (Charles Rivers, Hungary) ranging from 6-12 weeks of age used in this study were housed in the SPF animal facility of the Institute of Experimental Medicine, under regulated light-cycle with water and food supplied ad libitum. For embryo collection, 60-150 days old females were caged with males at late afternoons and checked for vaginal plugs next morning. The presence of a vaginal plug was considered as embryonic day 0.5 (E0.5). Pregnant mice, at various gestational days, were killed by cervical dislocation and embryos were recovered by Caesarean section.

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

2. CELL CULTURE

2.1. Preparing mouse embryo fibroblasts (MEF)

Primary culture of mouse embryonic fibroblasts was prepared using standard protocols. Briefly, 14-15 days after mating time pregnant females (C57Bl6) were killed by cervical dislocation and embryos were collected and washed in sterile phosphate buffer saline (**PBS**). Embryo head, limbs, and internal organs were removed under dissecting microscope in a sterile hood. Remaining embryo tissue was washed once in Dulbeco's Modified Minimum Medium (**DMEM**) and incubated for 45 min in 0.025% trypsin/EDTA solution at 37°C in a flask with gentle stirring. Subsequently, cell suspension was washed twice with DMEM supplemented with 10% fetal calf serum (**FCS**), centrifuged at 200g for 5 minutes and plated on tissue culture dishes ($5x10^6$ cells per 150-mm dish). After reaching confluence, cells were split 1:6 and

grown in culture for another 4 days. Exponential growing fibroblasts were frozen in the growing medium and kept under liquid nitrogen prior to use.

2.2. ES cell culture

Mouse embryonic stem cell line R1 (Nagy et al., 1993) were routinely grown on mitomycin C (100μg/ml, Sigma-Aldrich Co.) - treated or irradiated primary embryonic fibroblasts in high-glucose DMEM supplemented with sodium pyruvate, 20% heat-inactivated ES cell-qualified FBS, 2 mM L-glutamine, 1% non-essential amino acids (all from Invitrogen), 100 mm b-mercaptoethanol (Sigma-Aldrich Co.) and 1000 U/ml of leukemia inhibitory factor (ESGRO, Chemicon International). The cultured cells were checked visually, culture medium was changed daily and cells were passaged every 24-36 hours after plating. For passaging, medium was removed and cells were incubated in 0.02% trypsin in ethylenediaminetetraacetic acid (**EDTA**) for 5 min. Subsequently, trypsin was inactivated, cells were centrifuged at 1000g rpm for 5 minutes and plated on previously prepared fibroblast monolayer.

2.3. ES cell neuronal differentiation procedure

In vitro differentiation of ES cells to neurons was promoted with all trans-retinoic acid (RA) using modified 4-/4+ induction protocol (Bain et al., 1995). Briefly, after trypsinization fibroblasts were removed from ES cells by panning - three rounds of successive differential adherence steps to tissue culture dish. The remaining cell suspension (≤ 90% ES cells) was seeded in EB medium (ES medium without LIF and reduced serum to 15%) into bacterial grade culture dish allowing ES cells to aggregate and form embryoid bodies (EBs). Medium was changed every day by transferring the EBs to a 15-ml centrifuge tube; after sedimentation of the aggregates, the supernatant was replaced with fresh EB medium. At the end of day 4, growing medium was

replaced with a fresh one containing retinoic acid **(RA)**. The RA stock solution (10^{-2}M in dimethyl-sulphoxide – **DMSO**; Sigma-Aldrich) was diluted directly into the culture medium to obtain the desired concentration, $2x10^{-6}$ M. The medium containing RA was prepared in the dark and it was replaced every day.

At the eights day of induction period, intact or trypsinized aggregates were seeded on poly-L-lysine (20μg/ml; Sigma-Aldrich Co.)-coated tissue culture dishes or coverslips and twelve hours later medium was changed to DMEM supplemented with 10% FBS. This day was considered as "day 0 after induction" (dai 0). Four sets of experimental cultures generated at different time were used for this study.

2.4. Primary lens epithelial culture

The lenses were dissected from deeply anaesthetized newborn (P0) mice in ice-cold Tyroide buffer (Sigma-Aldrich Co.) with fine forceps under a dissecting microscope. Ten lenses were incubated in 500μl of 0.25% EDTA solution (Sigma-Aldrich Co.) at 37°C for 10 min. Following the addition of 1ml Medium 199, supplemented with 10% FBS, to slow the reaction, the suspension was triturated 10-20 times to dislodge cells from the lens capsula. Cells were pelleted at 200g for 2 min and plated on (approximately 2 lenses per 1.88 cm^2) matrigel–coated coverslip (BD Biosciences, 0.4 mg/ml) in growing medium. Medium was changed every 3-4 days and 2 hours before recordings.

Both primary cultures (fibriblast and LEC) and ES cells used in this study were kept at 37°C in a humidified atmosphere containing 5% CO_2.

3. GENE EXPRESSION ANALYSIS

3.1. *RNA isolation from cells and tissues*

Cells were washed twice with **DEPC** (diethyl pyrocarbonate, Sigma-Aldrich Co.) -treated PBS and lysed with TRI-reagent (1ml/90mm^2 surface, Sigma-Aldrich Co.) for 5min at room temperature (**RT**).

Lenses were dissected from embryos or deeply anaesthetized mice of various ages in ice-cold DEPC-treated PBS. The cilliary epithelium, vitreous and retina remnants were removed with fine forceps under a dissecting microscope. Lenses were collected in RNA-later (Sigma-Aldrich) and stored in the same solution at 4°C.

Embryonic and adult mouse brains were homogenized with TRI-reagent, and stored in liquid nitrogen.

Total RNA was isolated from cells and tissue using TRI-Reagent, following the manufacturer's protocol or by the method of Chomczynski and Sacchi (Chomczynski and Sacchi, 1987). Briefly, chloroform was added to the samples and mixed for 1min by inversion. After incubation at RT for 15 min, the samples were centrifuged at 12000 g for 15 min. The aqueous phase was transferred to a new tube and an equal volume of isopropanol was added. Carrier t-RNA or glycogen (20μg) was added to lens (E14.5, E15.5) samples to facilitate RNA recovery. The content was mixed for 1 min by inversion and then incubated at RT for 10 minutes or -20°C for overnight. RNA was recovered by centrifugation at 12000 g for 20 min. The pellet was washed in 70% ethanol, dried at RT and then dissolved in RNAse-free water. 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. The integrity of RNA samples were evaluated by running aliquots on 1.2% formaldehyde gels.

3.2. First-strand cDNA synthesis

Three micrograms of RNA were reverse-transcribed using RevertAid^{TM} H Minus First Strand cDNA Synthesis Kit (Fermentas) with random hexamer primers or Oligo-dT primers (Invitrogen) according to the manufacturer's instructions. 200 units of RevertAid^{TM} H Reverse Transcriptase or SUPERSCRIPT III (Invitrogen) and 0.5 μg of 12- to 18-mer primers were applied in 20μl mixture. Until use, reaction mixtures were stored at -20°C.

3.3. PCR amplification

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 12-40 cycles of amplification as follows: 60 sec at 94°C; 60 sec at 55- 68°C; 60-90 sec at 72°C followed by a final extension for 10 min at 72°C. For all RT-PCR assays the following control reactions were always carried out: (a) mouse β-actin was used as a positive control for the effectiveness of RNA extraction and RT-PCR reactions and as an internal standard for quantification; (b) direct amplification of RNA without reverse transcription served to exclude the presence of contaminating genomic DNA in the samples; (c) to test for contamination with exogenous DNA first strand cDNA template was replaced with water. Primer pairs for all studied genes were selected from different exons. Specific primers were also designed to identify all alternatively spliced transcripts encoded by the mouse GAD67 gene and to avoid the direct amplification of the GAD67 pseudogene may be present as contaminating DNA in the RNA samples. Sequences, annealing temperatures and extension times for each primer pairs are listed in Table 1. PCR products were separated on 1.5 -2% agarose gels containing ethidium bromide and run in **TBE** buffer (0.089M Tris, 0.089M boric acid and 0.002M EDTA). 100 bp DNA ladder (Fermentas) was used as marker to estimate the size of the amplified products.

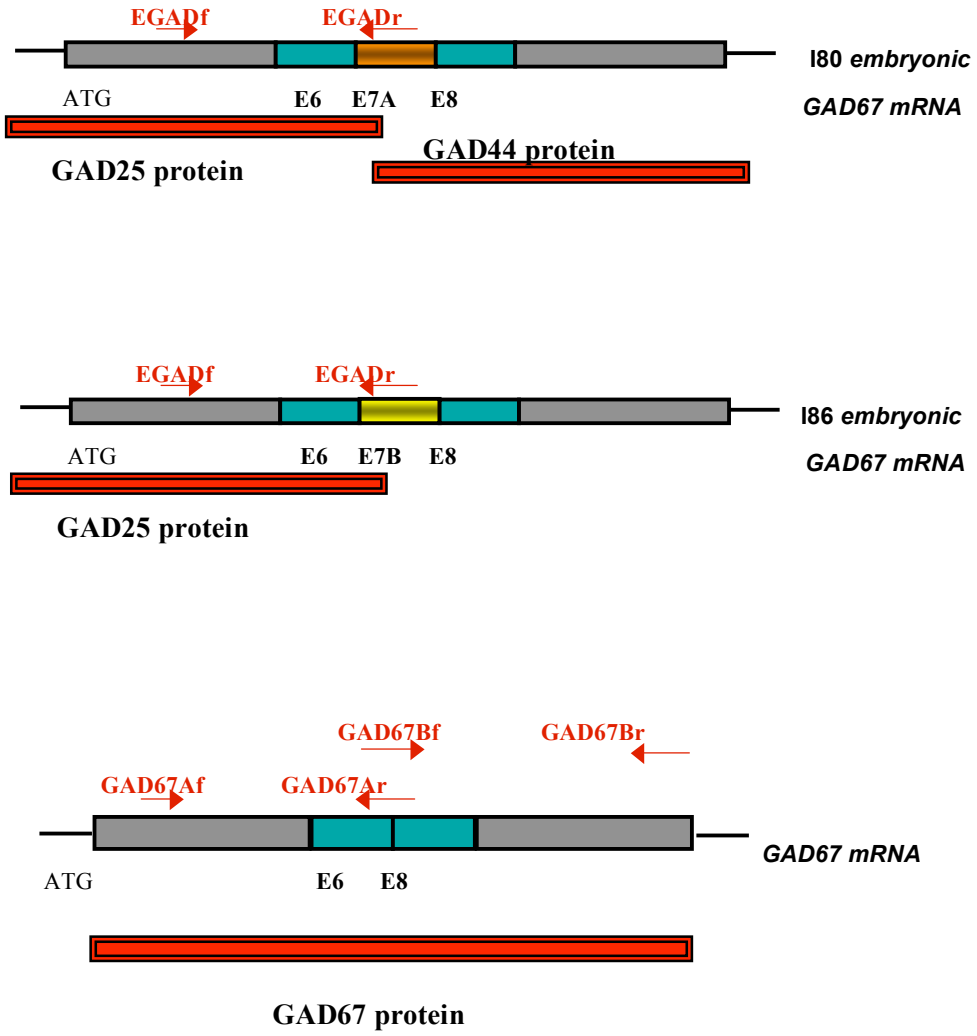


Fig. 11. Schematic representation of GAD67-family mRNAs with encoded proteins, and positions of PCR primers used for RT-PCR amplification. A1,A2. Embryonic GAD transcripts include either exon 7A (I-80, orange-filled box) or exon 7B (I-86, yellow-filled box). Primers used for PCR amplification are also indicated. Primer *EGADr* recognizes the embryonic exons (7A and 7B), while *EGADf* primer binds to an upstream region of GAD67 cDNA. This primer set co-amplifies both I-80 and I-86 transcripts. **B.** The embryonic exons (7/A and 7/B) are spliced out from the adult–specific GAD67 mRNAs, bringing together exon 6 and exon 8 (green boxes). We used two different primer pairs to amplify adult GAD67 mRNA (1) primer *GAD67Af* was selected from a region that is not represented in the GAD67-related pseudogene (Brilliant et al. 1990). Using this forward primer with corresponding reverse primer only GAD67 cDNA, but not contaminating genomic sequences derived from the pseudogene were amplified. Primer *GAD67Ar* spans a region shared by both exons 6 and 8 and therefore this reverse primer with the corresponding forward primer amplifies only the adult GAD67, but not the embryonic transcripts. (2) primer *GAD67Bf* also spans the border between exons 6 and 8 and *GAD67Br* binds to GAD67 coding region downstream of exon 8. Red boxes represent the protein products of different GAD mRNAs. Positions of the primer sets are indicated by arrows and their corresponding sequences are listed in Table 1.

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

mRNA	Position	Primer Sequence	Product Size (bp)	Anneal. Temp (°C)	Exten. Time (sec)	Ref.
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	*
GAD67A	618 1722	F 5'-GACTGCCAATACCAATATGTTCAC -3' R 5'-GGCGGCTGGGTTAGAGATG -3'	1105	60	90	*
GAD67B	85 659	F 5'-GATACTTGGTGTGGCGTAGCC-3' R 5'-ACCCATCTTCTGCTACAACCACTG-3'	575	62	90	*
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	Modified from (2)
$GABA_A\alpha2$	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	(3)
$GABA_A\beta1$	581 1386	F:5'-ATGGAGGAGAGGGAGCAGTAA-3' R:5'-AGAAAAGGTGATGGGGAAGAA-3'	806	55	90	(4)
$GABA_A\beta2$	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	(4)
$GABA_A\gamma1$	1077 1369	F 5'-CAACAATAAAGGAAAAACCACCAGA-3' R 5'-CCAGATTGAACAAGGCAAAAGC-3'	293	58	60	Modified from (2)
$GABA_A\gamma2$	553 964	F:5'-ATTGATGCTGAGTGCCAGTTGC-3' R:5'-TGGCTATGGTGCTTAAAGTTGTC-3'	412	60	60	*
$GABA_A\gamma2S$ $GABA_A\gamma2L$	1048 1362	F 5'-GTGGAGTATGGCACCCTGCATTATTTTGTC-3 R 5'-GATCCGAGCATAGGAGTCCATTTTGGC-3'	219+ 315	65	90	(5)
$GABA_A\gamma3$	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	*
GABAAε1 GABAAε2	1935 2393	F 5'-CAATGCGAAGAACACTTG-3' R 5'-TTAGCACGGCTATTGGTTGG-3'	459+ 381	55	60	(6)
$GABA_BR1$	859 1183	F:5'-ACGCATCCATCCGCCACAC-3' R:5'-AACCAGTTGTCAGCATACCACCC-3'	347	58	60	(7)
GABABR1a	161 441	F 5'-ATTTCCTGCCTGTGGACTATG -3' R 5'-GCCCTGACTACAGATGCTCC -3'	281	58	60	*
GABABR1b	88 572	F 5'-TCTCACTCCCCTCATCTCCC -3' R 5'-TTCTTCCAGCCCCACTTTTC-3'	485	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	(8)
GAT1	147 845	F:5'-ACGCTTCGACTTCCTCATGTCCTGC-3' R:5'-GAATCAGACAGCTTTCGGAAGTTG-3'	699	62	90	(9)
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'GCACCACCTCCCCGTCTTCGTTCTCCT-3'	572	68	180	(10)
NKCC1	2561 3309	5'-TGCGAGAAGGTGCACAATAC-3' 5'-TCTGTACGGCTCGATCATGT-3'	749	58	90	*
KCC2	4 402	5'-CTCAACAACCTGACGGACTG-3' 5'-ACAGAAGGACTCCATGATGCCCGCG-3'	399	62	90	*
Oct3/4	496 806	5'-GTTCTCTTTGGAAAGGTGTTC-3' 5'-ACTCGAACCACATCCTTCTC-3'	311	62	60	*
Nanog	59 297	5'-CTGGGAACGCCTCATCAA-3', 5'-CATCTTCTGCTTCCTGGCAA-3',	239	63	60	(11)
SOX2	240 790	F: 5'- GTGGAAACTTTTGTCCGAGAC-3' R: 5'-TGGAGTGGGAGGAAGAGGTAAC-3'	551	58	60	(12)
mTert	2079 2544	F:5'-ACCCAGGATGTACTTTGTTAAGG-3' R:5'-AGCAAACAGCTTGTTCTCCATGTC-3'	466	58	60	(13)
RyR1	1506 1861	F:-5′ CCACTTTGCCGAGTTTGC-3′ R; 5′-GGTCCTGGTTGGAGCGTA-3′	356	60	60	(14)
RyR2	2058 2390	F: 5′-GACGGCAGAAGCCACTCACCTGCG-3′ R; 5''-CCTGCAGAGAAACTGACAACTGG-3′	333	60	60	(14)
RyR3	210 482	F: 5′-GGAACCCACCTCAGAAGC-3′ R: 5'-TCCCGCAGACCTACATCA-3′	273	60	60	(14)
VGCC-L	3246 3336	F 5'-TCTTTATAAGGACGGCGATGTTG-3' R 5'- AGCCGAAAGGACATTGTCAAAA-3'	96	55	60	(15)
VGCC-N	3613 3740	F 5'- CGGGACCTGTGGAACATTCT-3' R 5'- CGCAGGACTCTCAGAGACTTGAT-3'	128	55	60	(15)
VGCC-R	5903 6035	F 5'- CTCTGGTGGTAACAGACCCTAGCT-3' R 5'- CTGCGCCGCGACTTG-3'	133	55	60	(15)
VGCC-T_G	2891 3166	F 5'-TGGAGGGTTTCCAGGCAGAG-3' R 5'-TGCTGGAGCTCTTGGGCAG-3'	276	55	60	*
VGCC-T_H	3106 3414	F 5'-GAGGAAGATTTCGATAAGCTC-3' R 5'-CTGTTCCAGCTGGAGCGC-3'	309	55	60	*
GIRK1	435 876	F 5'-CTATGGCTACCGCTACATCACAG-3' R 3'-CTGTTCAGTTTGCATGCTTCGC-3'	442	59	60	(16)
GIRK2	-97 5	F 5'- CGTGGAGTGAATTATTGAATCT-3' R 5'- GTCATTTCTTCTTTGTGCTTTT-3'	113	60	60	(17)

The positions are relative to the ATG codons of the corresponding cDNAs. Primer pairs for all studied genes were selected from different exons. F: forward, R: reverse,

(*) Gene-specific primers were designed for this study according to the published sequences as appeared in Ensembl (www.ensembl.org). (1) Varju et al., 2002 ; (2) Ma et al., 1993; (3) Liu et al., 1998; (4) Drescher, et al., 1993; (5) Homanics et al., 1999; (6) Kasparov et al., 2001; (7) Behar et al., 2001; (8) Chen et al., 2007; (9) Voutsinos et al., 1998; (10) Westmorelan et al., 2001; (11) Ginis et al., 2004; (12) Kania et al., 2005; (13) Amstrong et al., 2000; (14) Yu et al., 2008; (15) Etheredgeet et al., 2007; (16) Plummer et al., 2004; (17) Aguado et al., 2008.

3.4. Semi-quantitative RT-PCR analysis

For quantification of RNA expression, β-actin was generally co-amplified with the target gene for 20 cycles. When the target gene(s) and the β-actin were separately amplified due to the interference of the two simultaneous amplifications, the same master mix containing the first strand with the corresponding primers was used. RT-PCR reactions were performed in triplicate for each gene using RNA from two independent tissue preparations. In some cases the amplification reactions were trace–labeled with [32P] dCTP (3000Ci/mmol; Institute of Isotopes, Budapest, Hungary),

aliquots were run on 1.5-2% agarose gels, and then capillary blotted in 0.4MNaOH onto nylon membranes (Hybond-N+, Amersham Pharmacia Biotech, Buckinghamshire, England, UK). Blots were exposed and quantified with Phosphor Imager and values were normalized to β-actin. The results were averaged from triplicate reactions, and presented as ±SEM.

Quantification of data obtained by densitometry of ethidium bromide stained 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 each transcript were represented by using a gray scale or plotted in a histogram.

4. WESTERN BLOT ANALYSIS

Cells were harvested from the culture dishes in Ca^{2+}-. Mg^{2+}-free PBS containing 0.2%EDTA and homogenized in standard buffer (SB) containing 1mM aminoethylthioammonium bromide (AET), 0.2mM pyrodoxal 5-phosphate (PLP), 0.1mM phenylmethylsulfolyl fluoride (PMSF), 10mM benzamidine, 1mg/ml pepstatine, 1mg/ml leupeptin as protease inhibitors followed by brief sonication.

Lens protein extracts were prepared by homogenizing freshly isolated lenses in SB buffer containing 1 mM ethylene glycol tetraacetic acid (**EGTA**; Sigma).

Brain homogenates containing detectible amount of both embryonic and adult GAD forms, prepared from the newborn (P0) mice, were loaded to the same gels as controls.

Protein concentration was determined according to Bradford and samples were

adjusted to 2μg/μl in 2X Laemmli sample buffer (125M Tris-Cl, pH 6.8, 4 % SDS, 20% (v/v) glycerol, 0.2 M DTT, 0.02% bromphenol blue). Denatured protein samples were subsequently run in 10 or 12% sodium dodecylsulphate-polyacrylamide gels (SDS-PAGE; 20μg protein/lane) in mini Bio-Rad apparatus according to the manufacturer instructions.

Blotting was initially performed at 60V for 20 min, followed by 30 min at 80V and 5min at 100V to ensure blotting of larger proteins also.

The membranes were blocked and then were incubated overnight at 4°C with the primary antibodies diluted in TBST containing 1% bovine serum albumin (50 mM Tris±HCl pH 7.4, 100 mM NaCl; 0.05% Tween-20). The following antibodies were used: polyclonal rabbit sera no. 6799 (1/2000) recognizing all GAD forms also including GAD25 and GAD44 (Katarova et al. 1990; Szabo et al. 1994), polyclonal rabbit sera no. 8876 (1/1000) specific for GAD25 (Szabo et al. 1994) and monoclonal anti-βactin antibody (Sigma-Aldrich). After incubating with the appropriate alkaline phosphatase-conjugated secondary antibodies, blots were stained with NBT/BCIP (Katarova et al. 1990).

The molecular sizes of protein were estimated on the basis on molecular weights standards (BioRad) run on the same gels.

5. GABA MEASUREMENT BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

These experiments were performed in collaboration with Mária Baranyi from Department of Pharmacology, Institute of Experimental Medicine Hungarian Academy of Sciences.

Frozen lens tissue was homogenized in 50 μl 0.2 M ice-cold perchloric acid, cleared by centrifugation (3510 x g for 10 min at 4^{o}C) and neutralized by addition of 2M KOH (25:7 ratio). 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 previously described (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 was expressed per μg protein.

6. ELECTRON MICROSCOPY

Transmission electron microscopy sample preparation and photomicroscopy in collaboration with Dr. József Takács and Dr. Csaba Vastagh from Neurobiology Research Group, United Research Organization of the Hungarian Academy of Sciences and Semmelweis University .

ES cells grown for one day on a feeder layer in Petri dishes were fixed in 2% paraformaldehyde and 2% glutaraldehyde in 0.1 M PB pH 7.4 at RT for 20 min, then extensively washed and post-fixed with 1% osmium-tetroxide (0.1 M PB, pH 7.4) for 1 hour. All the following steps were carried out in the same plastic Petri dishes that cells were grown on, with cells fixed on the surface. Dehydration process was performed through serial incubation of graded series (50, 70, 90% and 100%) of ethanol. Cells were then sequentially infiltrated with mixture of 100% ethanol and epoxi resin (Durcupan™ ACM, Fluka) at various (2:1, 1:1, 1:2, repectively) ratios, followed pure resin, O/N. From the next day on, samples were incubated at 56°C for 72 hour for appropriate degree of polymerization. Series of ultrathin sections were cut

at 60-70 nm thickness and mounted on copper grids, counter-stained with uranyl-acetate and lead-citrate, finally examined and microphotographed in a transmission electronmicroscope at 80 kV.

7. IMMUNOHISTOCHEMISTRY

7.1. Tissue preparation for immunohistochemistry

Whole embryos or heads were fixed in 4% (w/v) PFA in PBS for 1 hr at RT or in 4% PFA-0.1% glutaraldehyde-PBS overnight at 4°C for GABA detection. The embryonic tissue was immersed in 20% sucrose-PBS solution at 4°C, 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 PBS containing 0.1% Tween-20 (**PBT**) 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.

7.2. GAD 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 and 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.

7.3. Immunofluorescent labeling

For immunofluorescent detection of GABA, $GABA_A\beta3$, $GABA_BR2$ receptor subunit, VGAT, GAT-1 and GAT-3, slides with serial cryostat sections were dried, washed with TBS for 5 min. They were subsequently blocked in 1% BSA, 0.02% saponin (Sigma-Aldrich Co.) in TBS for 2 hours at RT. The sections were incubated with primary antibodies: rabbit anti-GABA (Sigma-Aldrich Co., 1:5000), 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), diluted in 1% BSA, 0.005% saponin in TBS, overnight at 4°C. 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.) was applied for two hours at room temperature. Cell nuclei were labeled for 3min with 0.8 μg/ml DAPI (Sigma-Aldrich Co.). 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.).

8. IMMUNOCYTOCHEMISTRY

At various times after plating on coverslips, the cells were fixed in 4% PFA-0.1% glutaraldehyde in PBS (for GABA detection) or 4% PFA in PBS (for all other antibodies) for 20min at room temperature. Cells were permeabilized with 0.2% Triton X-100 for 5min and blocked in 0.5% blockig reagent (Roche Applied Science) or in 10% goat serum for 1-2 hours at RT. Cells were then incubated overnight at 4°C with primary antibodies in 1% BSA PBT or in TBST as follows: rabbit anti-GABA (Sigma-Aldrich Co., 1:5000), rabbit anti-GAD serum #6799 (1/1000, Katarova et al. 1990), rabbit anti GAD-65 serum N65 (1/500, Solimena et al. 1993), goat anti-αA-crystallin (Santa Cruz Biotechnology, 1:500), rabbit anti-αB-crystallin (Biomol International Inc., 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-cadherin, Sigma-Aldrich Co., 1:1000), mouse anti-N-cadherin (BD Transduction Laboratories, 1:1000), and goat anti-Oct4 (Abcam, 1:1000).

For light-microscope visualization, appropriate biotinylated secondary antibodies in dilution 1/500 were applied for 1.5-2 hours at RT, followed by incubation in with avidin-biotin complex in dilution 1/500 for 1hour at RT. Staining was visualized by

incubating the cultures in 0.05% 3,3'-diaminobenzidine (DAB) containing 0.003% hydrogen peroxide in 50mM TBS (pH 7.6). For fluorescence detection, fluorescin isothiocyanate (FITS)-conjugated anti-rabbit or Texas red-conjugated anti-mouse secondary antibody (both from Sigma Aldrich) was diluted in the blocking solution at 1/500 and were applied for 1 hour at RT. Cell nuclei were labeled for 3min with 0.8 μg/ml DAPI (Sigma-Aldrich Co.). In each experiment, control staining was performed following the same procedure but omitting the primary antibody.

Specimens 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, respectively.

9. $[CA^{2+}]_i$ IMAGING STUDIES

9.1. Measurements of change of intracellular Ca^{2+} concentration in the undifferentiated ES cells

ES cells line R1 grown for one day on a layer of primary embryonic fibroblast plated on the previous day on glass coverslips were loaded with 2.5 mM Fluo-4AM (Molecular Probes, Eugene, OR, USA) in perfusion buffer containing 0.2% pluronic acid (Sigma) for 45 min at 37°C. The buffer composition was (in mM): NaCl, 154; KCl, 5.6; $CaCl_2$, 2.3; glucose, 5.6; HEPES, 10, pH. 7.4. No specific measures were taken to remove fibroblasts from the cultures. Their distinct morphology allowed their identification, and Ca^{2+} measurements were only obtained within ES colonies. Cells were, mounted in a submerge type perfusion chamber onto the stage of an upright microscope (Olympus BX61WI,) equipped with the FluoView300 confocal laser-scanning system (Olympus). Serial scanning of cells was carried out at 488 nm excitation wavelength and fluorescence monitoring at 530 nm through a 20x water immersion objective. Whole field images were acquired every 3 s. Fluorescence

intensity was followed over time within regions of interest (ROI), covering a single ES cell. Fluorescence changes were expressed as the percentage of the average fluorescence value of the first 30 images. GABA (1mM; Sigma-Aldrich), $GABA_AR$ agonist muscimol (30μM: Sigma-Aldrich) and $GABA_BR$ agonist baclofen (20μM; Sigma-Aldrich) were applied in the absence and presence of the corresponding antagonists: bicuculline methiodide (20μM; Sigma-Aldrich), CGP 55845 or CGP 54626 (20μM; Tocris) in a randomised order. Cyclopiazonic acid (10μM; Tocris) and cadmium chloride (100μM; Tocris) were always applied after the application of the tested agonist alone.

9.2. Measurements of change of $[Ca^{2+}]_i$ in the lens cells

9.2.1. Intact lens

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, OR, USA). A stock solution containing 1 mM Fluo-4/AM, 20 % (w/v) pluronic acid in DMSO was prepared in advance. Subsequently the lenses were secured by means of a mesh to the bottom of a plastic perfusion chamber mounted on a laser scanning confocal microscope (LSM, Olympus FV-500). Recordings were made under excitation light emitted by an argon laser at 488 nm using a 10x optical and 5x digital magnification. Image acquisition frequency 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, Inc., WI, USA) 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 was applied for 2 min, followed by 2 min HBSS and finally HBSS-50 mM KCl. Analysis of fluorescence intensity was

performed using the imaging software of the Olympus FV500 (FluoView^{TM}).

9.2.2. Primary LEC culture

Primary LEC cultures were loaded with Fluo-4/AM (2.5 μM Fluo-4/AM in 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 Gmbh, Germany). 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 $GABA_A$R antagonist) or 20 μM of CGP55845 (Tocris Bioscience, UK; a $GABA_B$R 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 10x (1.3 NA) or 20x (0.7 NA) objectives of a laser confocal scanning microscope (Zeiss LSM 510 META, Carl Zeiss Inc., Germany) 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.

10. CELL PROLIFERATION STUDIES

For these experiments ES cells were plated at density of 1000/well on gelatin-coated 96-well assay plates (Corning, Inc). Twelve hours following cell seeding, cultures were treated with muscimol, SR95531, and baclofen, with final concentration 100, 50, 50μM, respectively and cultured for another 12 or 36 hours. Medium was replaced every 12 hours. Cellular proliferation was first measured using a fluorescence-based assay (CyQUANT Cell Proliferation Assay Kit; Molecular Probes, Invirogen) following manufactures's instructions. Fluorescence was measured by a multilabel microplate reader (Victor 3V; Per kin Elmer) using excitation and emission wavelengths of 480 and 550nm, respectively. A histogram was created by converting sample fluorescence values into percentage and normalized to the ones that were measured under control conditions (100%). Cellular proliferation: cell number and ES cells clone size were also determined after crystal violet (CV) staining and images of the entire growth area (from three different wells) were quantified in the ImageJ software (ImageJ; www.nihgov.com).

11. DATA ANALYSIS

Data are presented as either representative single experiments or mean ± S.E.M.. Statistical significance was estimated by unpaired two-tailed Studen's t test between control and treated cells (GraphPad InStat version 3.0a for Macintosh, GraphPad Software, San Diego California USA, www.graphpad.com"). The level of significance was expressed as follows: * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.0001$.

VI RESULTS

1. GABA SIGNALING IN THE MOUSE EMBRYONIC STEM CELLS

1.1. *Characterization of mouse ES cells, line R1*

1.1.1. *ES cells of line R1 express markers of pluripotency*

Pluripotency can be defined as the ability of a cell to differentiate into the multiple cell types of the embryo and adult (Solter, 2006; Niwa et al., 2007). In this study we worked with mouse embryonic stem cells line R1 (Nagy et al., 1993), widely used by many laboratories as they can contribute with high percentage to all tissues (including the germline) in chimaera production. In the presence of LIF and embryonic fibroblasts the undifferentiated ES cells form typical spindle- to round-shaped colonies (***Fig.12A***). The pluripotency of cells was monitored by the expression of Oct3/4, Nanog and Sox-2, key players in maintanance of the undifferentiated state in ES cells and early embryos (see Introduction) and the telomerase (mTert) which is highly expressed in stem cells but reduced upon differentiation (Amstrong et al., 2000). Our RT-PCR results showed that Oct3-4, Nanog, Sox-2 and mTert mRNAs were abundantly present in ES cells after one day in culture and nearly absent, with exception of Sox-2, in the newborn mouse brain (MB; ***Fig.12B***). Furthermore, immunocytochemical staining with Oct3/4 specific antibody revealed an intense and uniform nuclear labeling of all ES cell colonies (***Fig.12C***).

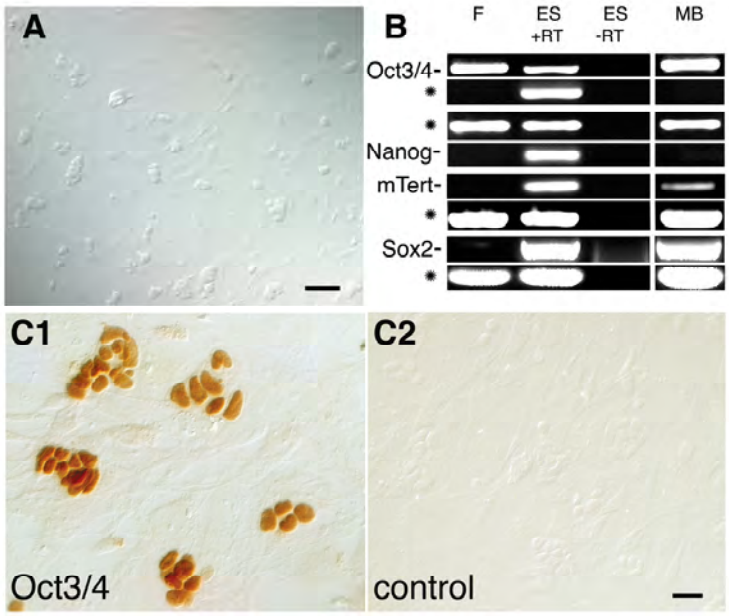


Fig.12. Mouse embryonic stem cells line R1 express markers characteristic for pluripotent phenotype. **A.** Phase-contrast image of one-day-old culture of ES cells grown on primary mouse fibroblasts. **B.** Expression of Oct3/4, Nanog, Sox-2 and mTert mRNAs in one-day-old R1 ES cell culture as revealed by semi-quantitative RT-PCR analysis. F- mitomicin-treated fibroblasts; ES- embryonic stem cells; - RT – reverse transcriptase omitted from first strand synthesis reaction; MB – mouse brain; * - actin; **C**. Images of ES cell culture immunostained for transcription factor Oct3/4. Scale bar: 100μm in A, 20μm in C1,C2.

1.1.2. *Ultrastructural features of cultured R1 ES cells show high level of similarity with other ES cells*

The fine structure of mouse ES cells was analyzed after one day in culture by transmission electron microscopy (TEM). All of the cells, appeared single or in compact colonies, had large nuclei and scanty cytoplasm with fewer organelles. The appearance of nuclei was mostly euchromatic with small regions of electrondense area of perinuclear heterochromatin (***Fig.13A***). Some of the ES cells were mitotic displaying characteristic midbody/interbody formations (***Fig.13B***). Furthermore, cells had close contacts with each other, connected by putative adherent and apparent gap junctions (***Fig.13D***). Occasionally we could detect clathrin-coated vesicles (CCV) near the membrane (***Fig.13C***).

These features are consistent with the ultrastructural characteristics of undifferentiated mouse ES cells (Niwa et al, 2007; Todorova et al., 2007).

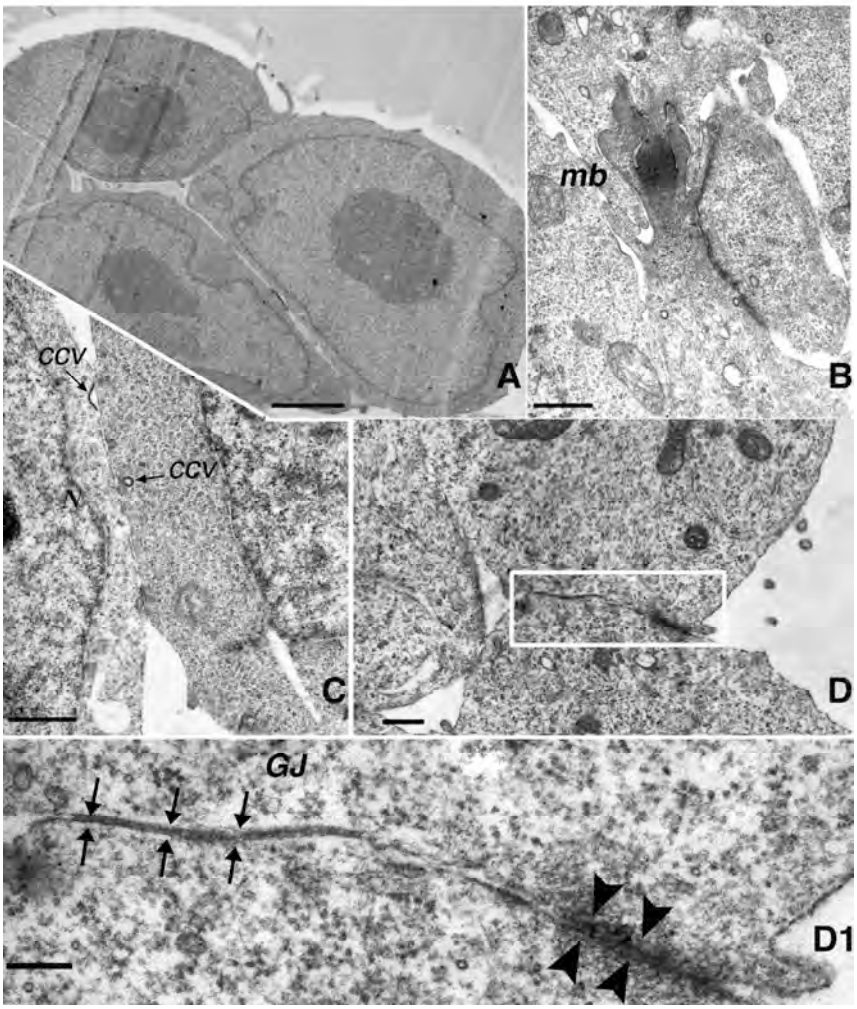


Fig.13. Ultrastructure of the undifferentiated ES cells. (**A**) Low magnification TEM image of an ES cell colony. Note the large mostly euchromatic nuclei.**(B)** Midbody/interbody formations between cells. **(C)** Clathrin-coated vesicles near the membrane. **(D)** Cells were tightly connected with apparent gap junctions (GJ) and adherent junction (arrows and arrowheads, respectively in enlarged image of boxed area in **D1**) mb, midbody; ccv, clathrin coated vesicle; GJ, gap junction. Scale bars: 2μm in A, 0.5μm in B, 500nm in C, 400nm in D and 200mn in D1. TEM study was done in collaboration with Dr. Joszef Takacs and Dr. Csaba Vastagh.

1.2. *Undifferentiated mouse ES cells express different GAD isoforms and contain γ-aminobutyric acid (GABA)*

Expression of different forms of GABA synthesizing enzyme GAD and their upstream transcription factors, Dlx2 and Dlx5, in the ES cells was examined by RT-PCR and immunocytochemistry. As outlined in the Methods Chapter, we identified the embryonic and adult transcripts encoded by the GAD67 gene, as well as the GAD65 transcript with specific primer sets. Adult GAD67 amplification was performed using a reverse primer spanning the exon 6-8 border region, thus

precluding amplification of exon 7A/B-containing embryonic cDNAs. For simultaneous amplification of the two embryonic GAD transcripts I-86 and I-80, (EGAD) reverse primer was derived from embryonic exon 7A/B. As shown in ***Fig.14A***, high mRNA levels of Dlx2, EGAD and GAD65 and only traces of Dlx5 and adult GAD67 transcript were found in undifferentiated ES cell but not in the embryonic fibroblasts - feeder cells.

The presence of GABA in ES cells was investigated by imunocytochemistry. As shown in ***Fig.14B*** relatively strong GABA immunoreactivity was observed in all ES cells while underlying fibroblasts were not labelled. All ES colonies showed consistently strong staining.

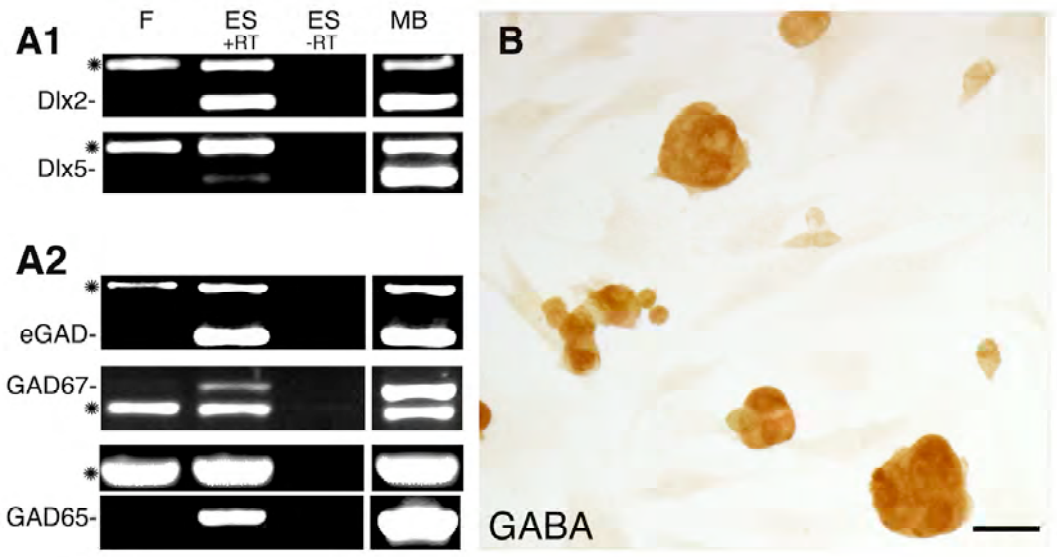


Fig.14. Expression of different Dlx and GAD forms and GABA in ES cells as revealed by RT-PCR (A1, A2) and immunocytochemistry (B), respectively. F- mitomicin-treated fibroblasts; ES- embryonic stem cells; - RT – reverse transcriptase omitted from first strand synthesis reaction; MB – mouse brain; * - actin; Scale bar: 20μm.

1.3. ES cells express both membrane and vesicular GABA transporters

We investigated the expression of all four membrane transporters (GAT1-4) and the vesicular GABA (VGAT) transporter in undiffrentiated ES cells both at RNA and protein levels using RT-PCR and immunocytochemistry, respectively. The results shown in ***Fig.15A*** demonstrate that only GAT-1 and VGAT are expressed in ES cells.

Furthermore, stainings with specific antibody demonstrated, especially in the case of GAT1, preferential membrane localization, although the fluorescence intensity varied from cell to cell (***Fig.15B***).

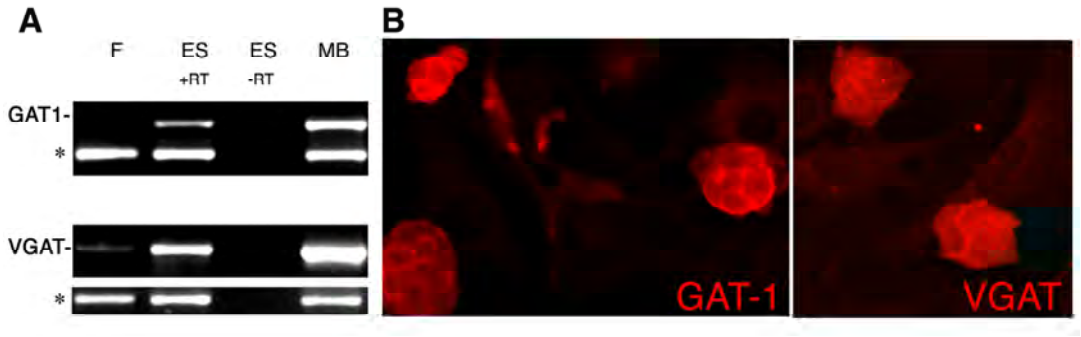


Fig.15. Expression of membrane and vesicular GABA transporters in undifferentiated ES cells, as revealed by RT-PCR (A) and immunocytochemistry (B). F- mitomicin-treated fibroblasts; ES- embryonic stem cells; - RT – reverse transcriptase omitted from first strand synthesys reaction ; MB – mouse brain; * - actin; Scale bar: 20μm.

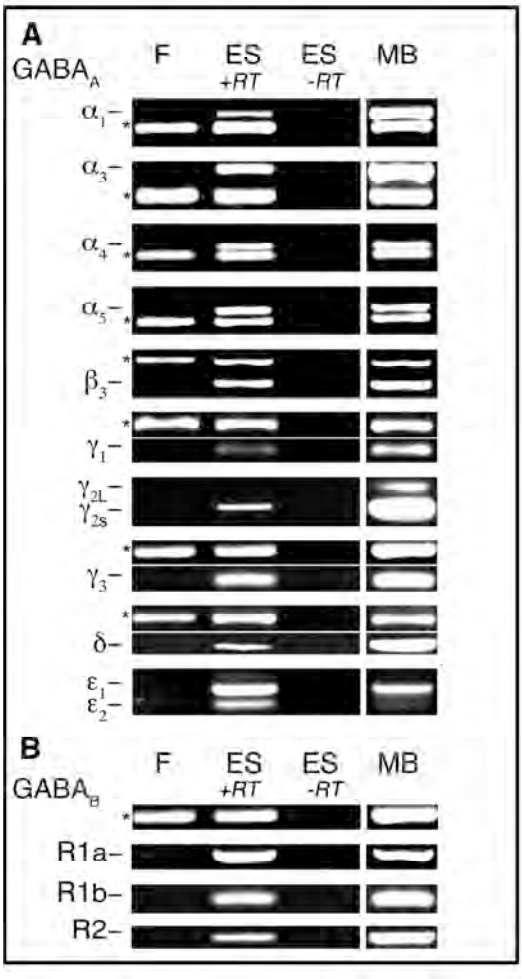
1.4. $GABA_A$ and $GABA_B$ receptor subunits are expressed in ES cells and transported to the cell membrane

First, we have studied the expression of different $GABA_A$ and $GABA_B$ receptor subunits in undifferentiated ES cells cultures by RT-PCR. We found that some, but not all, mRNAs for $GABA_A$ receptor ($GABA_AR$) subunit polypetides are expressed in undifferentiated ES cells. We could detect transcripts of α1, α3, α4,α5, β3,γ1, γ2S, γ3, δ as well as both splice forms of ε subunit in the ES cells, but not in fibroblasts. No α2, α6, β1 or β2 transcripts were detected even after 35 amplification cycles. Noteworthy is the presence of the shorter version of γ2 subunit - γ2S (***Fig.16A***) and the absence of γ2L. Moreover, mRNAs coding for the $GABA_C$ receptor subunits (ρ1 and ρ2) were not detected in the ES cells.

We found also abundant expression of both $GABA_BR1$ subunits ($GABA_BR1a$ and $GABA_BR1b$,) and $GABA_BR2$ (***Fig.16B***), therefore providing the structural basis of a functional receptor assembly.

The ratio between R1a and R1b variants showed opposite tendency compared to mouse brain - there is a clear prevalence of the sushi domain- containing R1a subunit in ES cells, whereas the shorter R1b is the predominant form in the brain (Fritschy et al., 1999). These results were highly reproducible in independent experiments using RNA obtained from different ES cell cultures.

For immunofluorescent detection of $GABA_A$ receptors in the ES cells, we used an antibody that recognizes the GABA binding β3 subunit of the $GABA_A$ receptor, a component of most abundant functional GABA receptors in the developing and mature brain. ***Fig.17a*** shows β3 immunolabeling in all ES cells with more intense staining at the borders of neighboring cells (arrows).



The subcellular distribution of $GABA_B$ receptors in ES cells was examined by immunofluorescent labeling of cultures with $GABA_BR2$ specific antibody, as this subunit is shown to be essential for correct trafficking of the receptor to the cell membrane and interaction with its cognate G protein. As shown ***Fig.17b*** positive staining was observed in all ES cells, while underlying fibroblasts were not labeled. High magnification revealed a pattern of bright "vesicular" appearance along the border of neighboring cells.

Fig.16. Expression of $GABA_A$(A) and $GABA_B$ (B) receptor subunits in undifferentiated embryonic stem cells as revealed by RT-PCR. F- mitomicin-treated fibroblasts; ES- embryonic stem cells; - RT – reverse transcriptase omitted from first strand synthesis reaction; MB – mouse brain; *-actin.

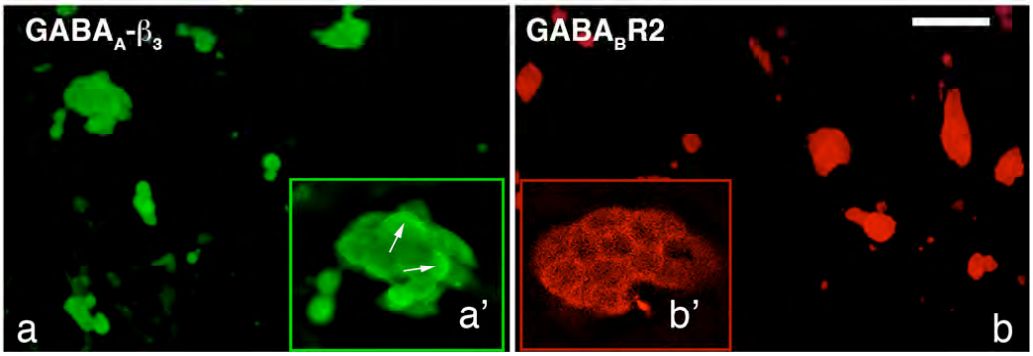


Fig.17. Immunfluorescent localization of GABA$_A$-β3 (a) and GABA$_B$R2 (b) receptor subunits in mouse ES cell colonies. Insets a' and b' represent enlarged single clones of ES cells from a and b, respectively. Arrows in a' and arrowheads in b' mark the intensive signal at cellular borders. This enrichment of GABAR subunits at sites of putative contacts of adjacent cellular membranes suggests that both types of GABAR are engaged in intensive cell-cell communications within the ES colony. Scale bar: 50μm

1.5. *ES cells express functional GABA$_A$ and GABA$_B$ receptors*

In order to test the functionality of the GABA receptors, we examined the effect of receptor activation on the intracellular calcium levels, since we postulated that GABA action on ES cells could be mediated though calcium signals similar to immature neurons.

1.5.1. *Expression of calcium signalig related molecular components in ES cells*

The regulation of calcium (Ca^{2+}) signaling depends on the interplay between intracellular and extracellular calcium sources (Beridge et al., 2003). Several molecules related to calcium signalig such as IP3 receptor (IP3R)-regulated intracellular Ca^{2+}-stores, store-operated Ca^{2+}-channels, and both plasma membrane Ca^{2+}-pumps and Na^{+}/Ca^{2+}-exchagers have been described recently in undifferentiated ES cells (Yanagida et al., 2004; Kapur et al., 2007). Several other components, including G protein couple inwardly rectifying potassium channels (GIRK1 and GIRK2) and voltage gated calcium channels (VGCC) have not yet been identified in ES cells, however they are known to be involved in GABA induced Ca^{2+} signaling in

the immature neurons (Owens and Kriegstein, 2000). We have checked for the presence of the above molecular componets at mRNA level in undifferntiated ES cells. As shown in Fig.18, we could detect by RT-PCR the transcripts encoding Kir 3.1(GIRK1) and Kir3.2 (GIRK2) potassium channels (***Fig.18A***) as well as L, N, R, and T-type Ca^{2+}-channels (***Fig.18B***) in ES cells. Moreover, mRNA for two out of three members of ryanodine receptors, RyR1 and RyR2, mediators of intracellular calcium stores-dependent calcium efflux in early stages of embryonic development (Rossi and Sorrentino, 2002), were also detected (***Fig.18C***).

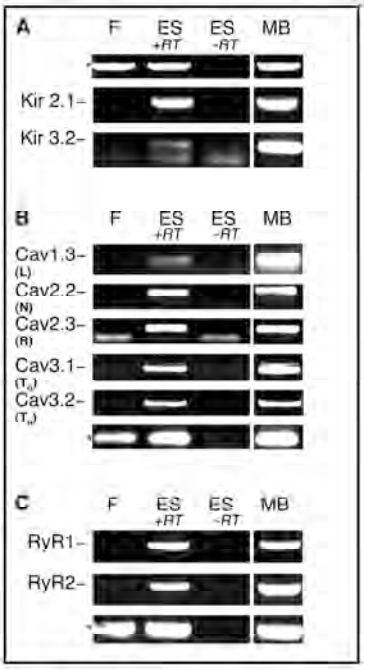


Fig.18. Expression of different inwardly rectifying potassium channels (A), voltage–gated calcium channels (B) and ryanodine receptors (C) in undifferentiated ES cells as revealed by RT-PCR. F- mitomicin-treated fibroblasts; ES- embryonic stem cells; -RT – reverse transcriptase omitted from first strand synthesis reaction ; MB – mouse brain; * - actin;

1.5.2. *Activation of $GABA_A$ and $GABA_B$ receptors triggers rise in the intracellular calcium levels in ES cells*

The presence of multiple subunits of $GABA_AR$ and the two subunits of $GABA_BR$ in undifferentiated ES cells indicated that the assembly of functional receptors is possible. To test this assumption we monitored the changes in $[Ca^{2+}]_i$ by confocal calcium imaging in ES cells upon application of GABA and selective receptor agonists/antagonists.

After 16-18 hours in culture, ES cells formed multicellular colonies exhibiting a distinct round boundary without signs of outgrowth. After loading of the Ca^{2+}-sensitive dye Fluo-4AM, we monitored in the presence of GABA or selective $GABA_A$ and $GABA_B$ receptor agonists/antagonists the changes of fluorescence intensity in individual cells within a colony, which correspond to changes of $[Ca^{2+}]_i$. Addition of GABA to the recording chamber (1 mM) for 90 s induced a sharp rise in $[Ca^{2+}]_i$ in a significant number of cells (198.99±47.85 %, n = 38 in 13 coverslips; ***Fig.19***.).

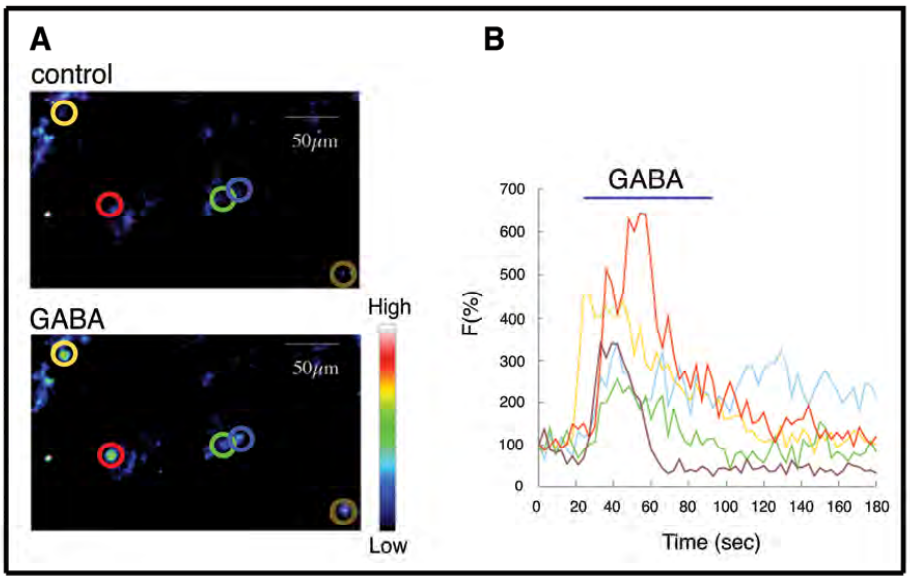


Fig.19. GABA induces calcium transients in undifferentiated mouse ES cells. **A:** Confocal images were taken at different time points of the recording shown in panels B. B2 Representative recording of GABA evoked changes in fluorescence of Fluo4/AM-loaded ES cells **B:** Graph that shows changes in fluorescence intensity in cells circled on images in A panel. F(%): the change in intracellular Ca concentration is given as the change in fluorescence relative to the basal fluorescence at the beginning of experiment, which was normalized to 100%.

To characterize the GABA receptor type linked to the $[Ca^{2+}]_i$ increase evoked by GABA, the effect of selective GABAR agonists and antagonists were tested. As expected, muscimol (30 μM) a selective $GABA_A$ receptor agonist induced sharp rise of $[Ca^{2+}]_i$ (n = 31 in 14 coverslips) with a 147,98±26,6% increase in the amplitude of the response. This effect was reversally blocked by the selective $GABA_A$ receptor antagonist, bicuculline (20μM) (n=0 in 14 coverslips, $p<0.05$), confirming that the effect of muscimol is due to $GABA_A$ receptor activation (***Fig.20: A,D***).

To test whether the $[Ca^{2+}]_I$ increase induced by activation of the $GABA_AR$ was due to Ca^{2+} release from intracellular Ca stores or/and influx through VGCC, we applied specific inhibitors to block each pathway.

Application of sarcoplasmic-endoplasmic reticulum Ca^{2+}-ATPase blocker CPA (10 μM) resulted in Ca^{2+} transients when it reached to the perfusing chamber (n = 26 in 7 coverslips) and after 120.3±4.2 s-long incubation activated a capacitive Ca^{2+} entry (n = 15 in 7 coverslips, and data not shown), therefore the effect of muscimol was tested 60 s after CPA was applied. In the presence of CPA the occurence of muscimol-evoked Ca^{2+} transients was greatly reduced ($n = 4$; $p < 0.05$), while their amplitude remained the same (126.55±48.11 %; ***Fig.20: B,D***).

Initial bath application of the voltage–gated calcium channel (VGCC) blocker cadmium (100 μM) significantly reduced the amplitude of the Ca transients (by 37%) but not the number of cells displaying calcium transients induced by subsequent addition of muscimol (93.14±13.68; 1.83±0.31; n=6; ***Fig.20: C,D***).

The above findings show that $GABA_A$ receptor activation evokes calcium release from internal stores and influx through VGCC.

Strikingly, $GABA_BR$ agonist baclofen (20μM) evoked a sharp and significant increase in fluorescence in ES cells (2.70±0.32, n = 23; 177.35±29.88 %), that, in most cases, was wave-propagated to neighboring cells (***Fig.21: A,E***). Surprisingly, we could not block the Ca^{2+} transients induced by baclofen with two known $GABA_BR$ antagonists- CGP 55845 (20 μM) or CGP 54626 (10 μM), which are widely used to block neuronal $GABA_BR$ signalling. CGP 55845 affected neither the number of cells showing baclofen-induced $[Ca^{2+}]_i$ increase nor the amplitude of the evoked transients (3.0±0.71 and 210.84±47.73, respectively; $n = 5$, $p > 0.05$; ***Fig.21: A,E***).

The amplitude of the calcium transients, induced by the application of KCl were further increased by addition of baclofen (***Fig.21B***) supporting the notion that in

contrast to neurons, the predominant effect of $GABA_BR$ activation in undifferentiated ES cells is $[Ca^{2+}]_i$ rise.

$GABA_BR$ have previously been shown to suppress (Bowery et al., 2002) or facilitate Ca^{2+} influx via several sub-types of VGCCs (Parramon et al., 1995; Carter and Mynlieff, 2004). Pretreatment of ES cultures with Cd^{2+} (100μM) a potent blocker of the VGCC reduced the amplitude of the baclofen-induced $[Ca^{2+}]_I$ transients, but not the number of responding cells (91.03±14.92 and 3.2±0.2, respectivelty, n=5, ***Fig.21: C,E***). Furthermore, baclofen-evoked Ca^{2+} responses were essentially unaffected when Ca^{2+} -free buffer was used (2.33±0.67; 206.12±53.94; n=3), which clearly demonstrates that $GABA_BR$– induced Ca^{2+} transients are largely independent of extracellular Ca^{2+} (***Fig.21: D,E***).

In contrast to muscimol-induced calcium signals, those induced by the baclofen were nearly completely omitted in the presence of the endoplasmic-sarcoplasmic reticulum Ca^{2+}-ATPase blocker CPA (0.33±0.33; $n = 3$ $p < 0.05$; ***Fig.21E***), indicating they originate from intracellular calcium stores.

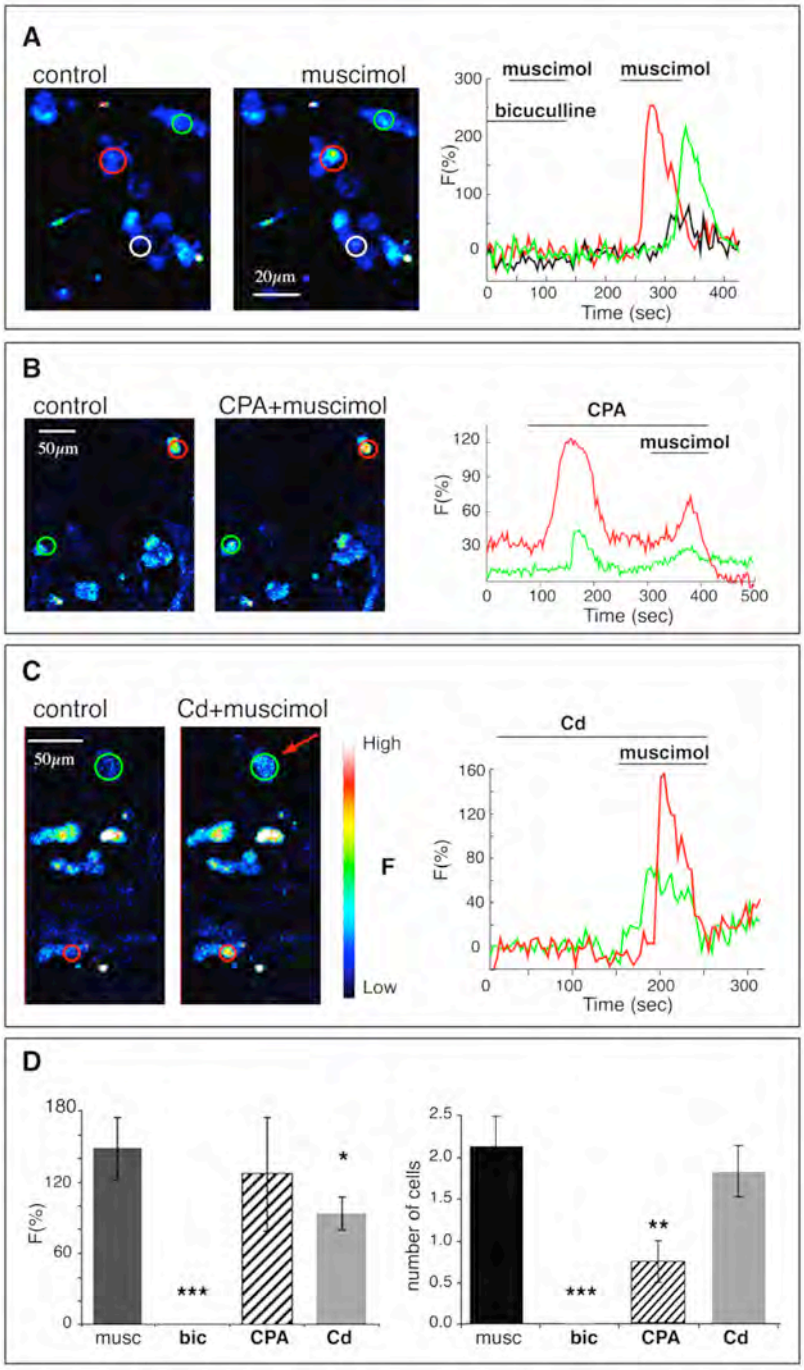


Fig.20. Modulation of agonist-induced $GABA_A$ responses in ES cells. A: Representative recordings of $GABA_A$ receptor-evoked changes in fluorescence of Fluo4/AM-loaded ES cells. Cultures were initially perfused with 30 μM bicuculline, then 30 μM muscimol and 20 μM bicuculline were co-administered followed by application of 30 μM muscimol (Muscimol) alone **B:** Effect of intracellular Ca^{2+} store blockage on muscimol-induced rice in $[Ca^{2+}]_i$ in ES cells. Cells responded to muscimol in the presence of cyclopiazonic acid (CPA), but with lower amplitude. **C:** Example of muscimol-induced $[Ca^{2+}]_i$ in ES cells after 3min pre-incubation with VGCC blocker cadmium (**Cd**). **Left panels:** Cells manifesting $[Ca^{2+}]_i$ transients are circled on the confocal images taken at different time points of the recording. **Right panels:** Change in fluorescence intensity (**F(%)**) in cells circled on corresponding images.**D**: Comparison of $GABA_A$ induced $[Ca^{2+}]_i$ responses (**musc**) in the presence of bicuculline (**bic**), SERCA blocker CPA (**CPA**) and VGCC blocker cadmium (**Cd**). Bar charts illustrating the amplitude of the responses (**F(%)** (left panel) and the average number of cells responding to the treatment (right panel). **F(%):** the change in $[Ca^{2+}]_i$ is given as the change in fluorescence relative to the basal fluorescence at the beginning of experiment (100%).

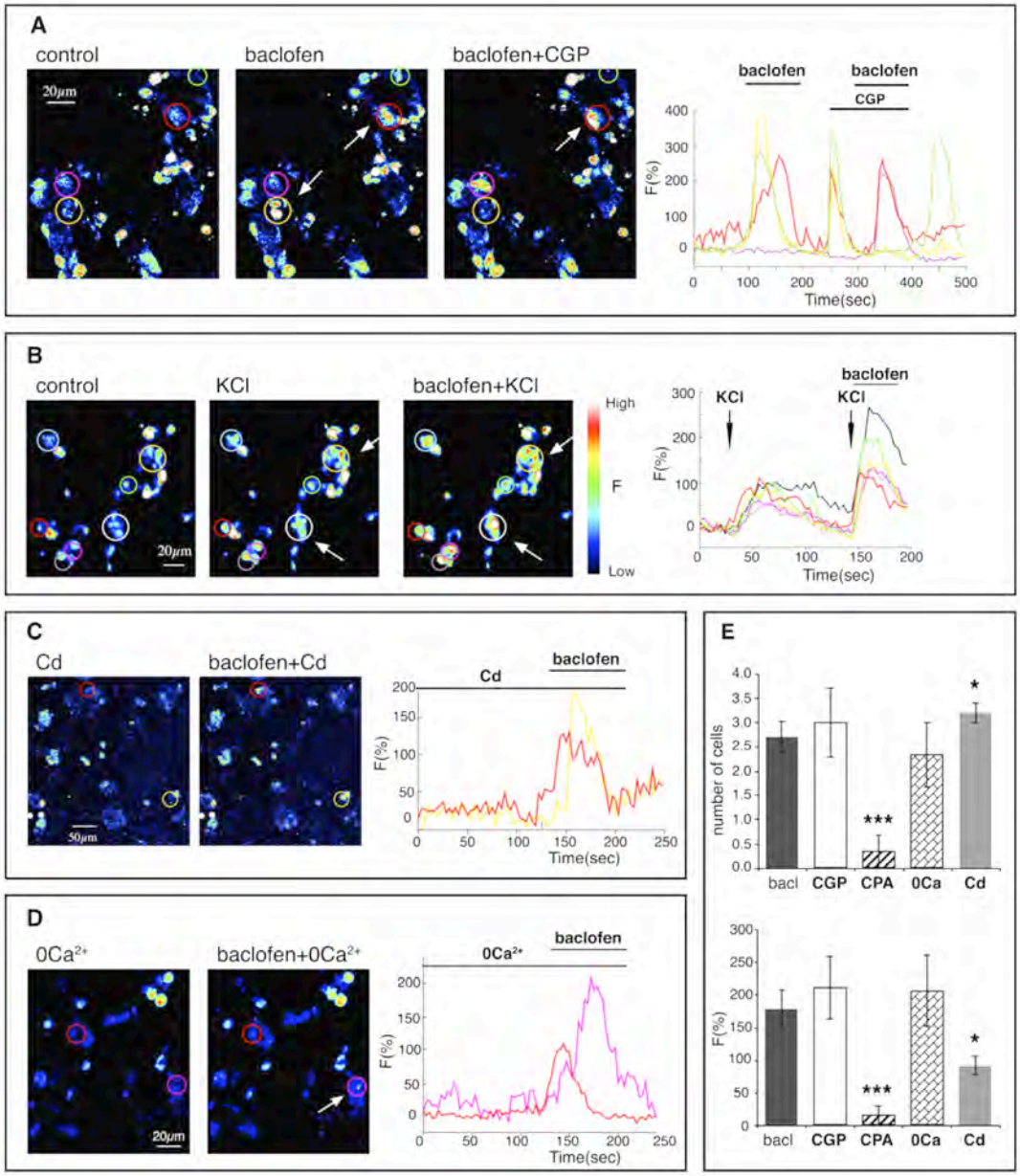


Fig.21. Modulation of agonist-induced $GABA_B$ responses in ES cells A: Representative recordings of $GABA_B$ receptor-evoked changes in fluorescence of Fluo4/AM-loaded ES cells. Cultures were initially perfused with 30 μM baclofen, then 20μM CGP and finally bacofen and CGP were co-administered (baclofen plus CGP). **B:** Seven sample mean time courses of $GABA_B$ - induced changes in $[Ca^{2+}]_i$ in ES cells after addition of KCl (**KCl**) followed by perfusion with KCl-baclofen (**KCl + Baclofen**); note that baclofen further increased the KCl-evoked responses. **C:** Example of baclofen-induced calcium transients in ES cells after 3min pre-incubation with VGCC blocker cadmium (**Cd**). **D:** Example of baclofen-evoked Ca^{2+} responses in Ca^{2+}-free buffer. **Left panels:** Cells manifesting $[Ca^{2+}]_i$ transients are circled on the confocal images taken at different time points of the recording. **Right panels:** Change in fluorescence intensity (**F(%)**) in cells circled on corresponding images. **E:** Bar charts comparing the amplitude of the responses (left panel) and the average number of cells responding to the treatment (right panel). The change in intracellular Ca concentration is given as the change in fluorescence relative to the basal fluorescence at the beginning of experiment (100%).

1.6. *GABA receptors modulates ES cell proliferation*

Previously, it has been shown that GABA and different GABA receptor ligands can modulate the proliferation of neural stem cells of the embryonic ventricular zone (VZ) (Lo Turco et al., 1995; Haydar et al., 2000, Fukui et al., 2008). To test the effect of GABA activation on ES cell proliferation we plated ES cells at low numbers in multi-well plates, grew them under standard conditions for 18 hours to allow for stable membrane expression of the GABA receptor, thereafter added different agonists/antagonists to ES growth medium. It should be stressed that omitting of either LIF or serum from the medium, or use of dialyzed serum for prolonged incubation without application of drugs had an inhibitory effect over the ES cell growth and proliferation (data not shown).

Cell numbers were estimated 24/48 hrs following addition of the drugs using two different approaches. In proliferation tests, no statistically significant differences in the fluorescence intensity (proportional to cell number; see Materials and Methods) were encountered after one day-treatment with the muscimol, SR95531, baclofen (***Fig.22Aa***) or CGP (not shown). Exception was the $GABA_AR$ blocker bicuculline, which proved highly toxic to the ES cells at concentration applied for blocking the $GABA_A$ receptor (10-30μM), therefore we excluded it from later analysis and used instead the highly specific $GABA_AR$ antagonist SR95531. Treatment of ES cells with these drugs for a further 24 hrs revealed a much more complex picture. While the $GABA_AR$ agonist muscimol slightly, but not significantly decreased ES cell numbers compared to control, the $GABA_BR$ agonist baclofen greatly increased the total cell numbers and size of clones. The effect of simultaneous treatment with both muscimol and baclofen was cumulative of the two drugs alone, i.e. cell numbers were close to control. Addition of the highly specific $GABA_AR$ antagonist SR95531 to the media produced the strongest proliferative effect by nearly doubling the cell number

(average clone size) compared to control (***Fig.22Aa***). These results indicate that GABA affects proliferation of undifferentiated ES cells acting through both, $GABA_A$ and $GABA_B$ receptors.

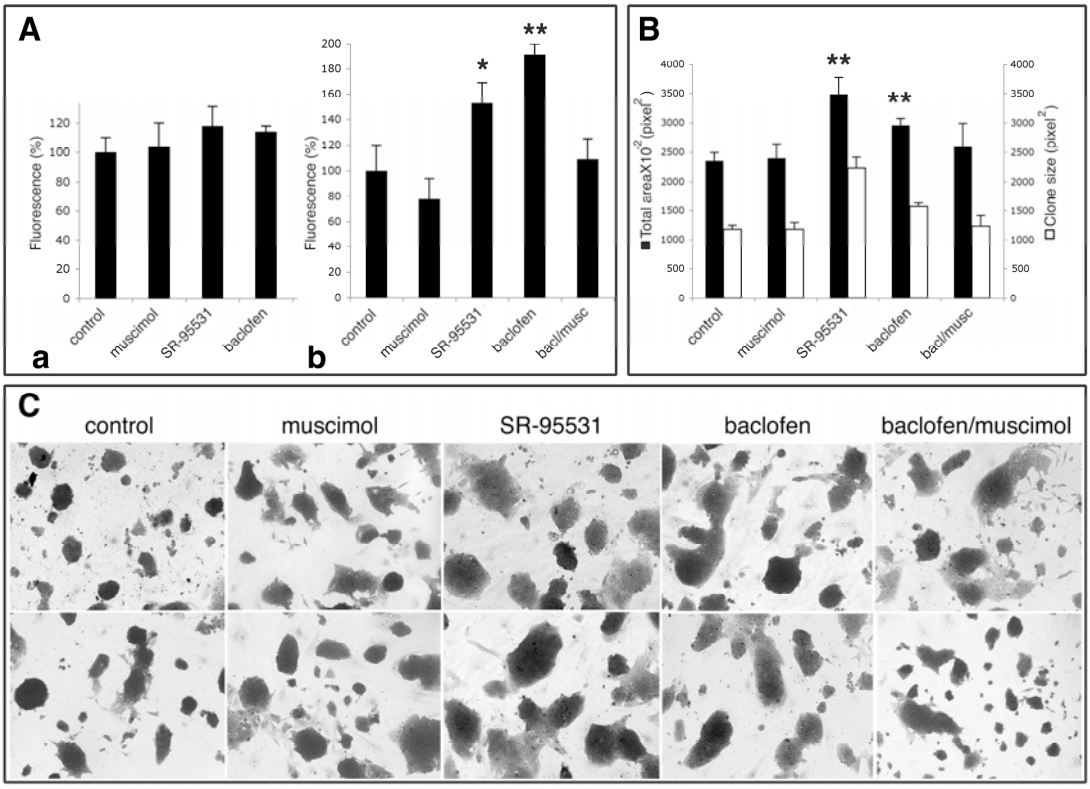


Fig.22.. Effects of selective GABAR agonists and antagonists on the proliferation of undifferentiated ES cells. A: ES cells were incubated in the standard ES medium supplemented with 100 mM muscimol (Muscimol), 50 mM SR-95531 (SR-95531), 50 mM baclofen (Baclofen) or baclofen plus muscimol (Baclofen/Muscimol) for 18(a) and 24(b) hours and cell viability was determined using a fluorescence-based assay (CyQUANT Cell Proliferation Assay Kit; Molecular Probes, Invitrogen). Fluorescence in ES medium without additional drug treatment (Control) was considered to be 100%. **B, C:** Cellular proliferation was determined by measuring the size and total area of ES clones, which correspond to cell numbers. Cells were visualized with cresyl violet, images were captured with digital camera (Zeiss) and evaluated using NIH image software. Bar charts (B) and representative whole-field images of cells (C) treated as described above.

2. SEQUENTIAL EXPRESSION OF MOLECULAR COMPONENETS OF GABA SIGNALLING DURING IN VITRO NEURONAL DIFFERENTIATION OF MOUSE EMBRYONIC STEM CELLS RECAPITULATES EMBRYONIC DEVELOPMENT

In the present study, we examined the expression dynamics of different GAD forms GABA receptor subinits and GABA transporters during in vitro neuronal differentiation of mouse embryonic stem cells. We also characterized GABA synthesizing cells by immunocytochemistry. Our results demonstrate that neuronal progenitors synthesize different forms of GAD and contain GABA during earliest days of in vitro differentiation. Furthermore, the temporal expression profile of molecular components of GABA signalling machinery was highly consistent in our experiments and resembled its embryonic counterpart.

2.1. *Expression of different GAD isoforms during in vitro differentiation of ES cells*

According to the currently used induction protocol for *in vitro* neuronal differentiation (4-/4+; Bain et al., 1995), ES cells are initially cultured in suspension, in the absense of LIF, to induce ectodermal differentiation. The subsequent addition of high concentration of retinoic acid (RA; 10^{-7}-10^{-6}) to the ES cell agregates (embryoid bodies; Yamada et al., 1994; Leahy et al., 1999), induces rapid neuronal, and repress mesodermal gene expression in cells (Bain et al., 1996).

We have analysed the temporal expression pattern of different mRNAs encoded by GAD and Dlx genes during defined phases of ES cell neuronal differentiation by semi-quantitative RT-PCR analysis. Total RNA was isolated from ES cells, cell aggregates grown in suspension for 8 days (EB; 4-/4+ RA), and from attached EBs differentiated for 2, 4, 6, 8 and 10 days following induction period. The average of gene expression, rellative to the actin expression, was calculated from at least three RT-PCR obtained from three independent in vitro differentiation experiments.

We detected a considerably high level of EGAD transcript in cells from all stages that was slightly increased on day 4 (dai4; ***Fig.23***). In contrast, GAD67 mRNA was first detected in the cells grown for two days following RA (dai2) and gradually increased during maturation. Using the same PCR protocol condition as in the case of other genes, the expression of GAD65 mRNA was first seen in cells from one week old cultures (dai6), thereafter increased exponentially as they proceeded toward neuronal differentiation (***Fig.23***). However, GAD65 transcript could be amplified from ES cells (see Chapter 1.2.) to dai4 stage (not shown) after 35 amplification cycles.

In our cultures, both genes, Dlx2 and Dlx5 were expressed transiently, with partially overlapping patterns.The Dlx2 transcript was expressed, at a considerably high level, in ES cells and EBs, then increased between dai2-dai4, and decrease after dai6, being undetectable beyond the 8th day after RA-induction. Expression of Dlx5 mRNA was detectable for the first time at dai2, slightly increased by dai4 and remained practically unchanged until dai8 after which sharply declined.

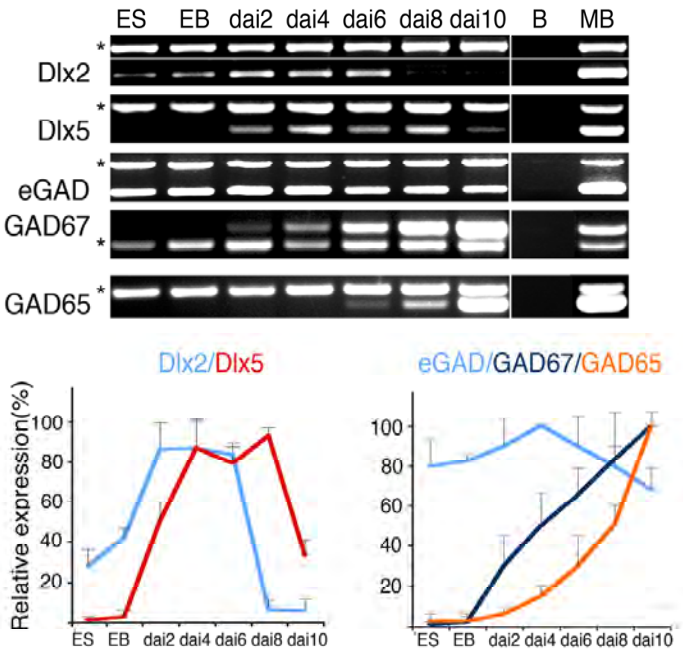


Fig.23. Temporal expression patterns of Dlx2, Dlx5 and GAD gene family in ES cells toward neuronal differentiation as revealed by semi-quantitative RT-PCR. **A**: Representative RT-PCR amplifications of Dlx2 and Dlx5 and different GAD (EGAD, GAD67 and GAD65) transcripts using cDNA synthesized from total RNA preparations of undifferentiated (**ES**) and RA-induced ES cells (**EB, dai2-dai10**), and adult brain (**MB**). Expression levels were normalized to β-actin,(*) and represented on a grey scale as % of max level. **B**: Plots (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%).

The GAD proteins were identified on Western blots of cell homogenates from ES cells and in RA-induced ES cells. The GAD25 protein, previously shown to run as

two closely spaced bands (32-32kD), (Szabo et al,1994), were expressed, at considerably high level in ES and EBs, up-regulated from dai2 to dai8 and thereafter declined but still detectible at dai 10 and beyond showing similiatities to embryonic expression (***Fig.24***). The GAD44 protein was expressed at very low level in ES cells, but then increased gradually and remained stabily expressed throughout the studied period. The translated GAD67 and GAD65 were first detected, at the protein level, at later differentiation periods, around dai6 and dai8, respectively, which matches with the RT-PCR results.

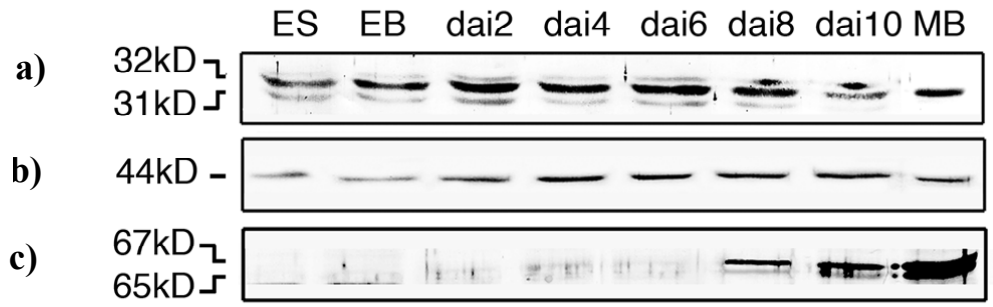


Fig.24. Western blot analysis of GAD forms toward neuronal differentiation of ES cells: protein homogenates from cells isolated from the defined stages of neuronal differentiation (EB, dai2-dai8) were run on 12% SDS-PAGE, blotted and stained with an anti-GAD rabbit serum #8876 (a) and #6799 (b,c).

2.2. *Expression of different GABA receptor subunits and GABA transporters*

We found all examined $GABA_A$ receptor subunits to be expressed during *in vitro* neuronal differentiation of ES cells. The mRNAs for α3 and α5 subunits could be detected already in ES and EBs, whereas α2,α4, and α1 subunits were detectible after induction of neuronal differentiation (dai2), respectively (***Fig.25***). The β1 subunits showed a low level of expression in the EBs, and gradually increased from day 4, while β2 could be detected for the first time at dai2 with reasonable high level of expression that stayed practically unchanged until day 10. β3 transcript, which is the predominant β subunit expressed during embryonic development (Laurie et al.,1992; Wisden et al.,1992; Serafini et al.,1998) had high level of expression in ES cells and

EBs and remained constant until dai10 (***Fig.25)***. γ1 and γ2L expression paralleled the neuronal differentiation from dai2 on. γ2S and γ3 mRNA displayed relatively high levels of expression at all stages and was increased after RA-treatment.

Using specific primer pairs, designed for each subunit type, we investigated the expression of $GABA_BR1$($GABA_BR1_a$ and $GABA_BR1_b$) and $GABA_BR_2$ receptor subunits during neuronal differentiation. All three mRNAs were detected already in ES cells and EBs and gradually increased during differentiation (***Fig.25***). The sushi-domain containig $GABA_B$R1a subunit is the most abudant of all three.

Next, we examined the dynamics expression of different plasma membrane (GATs) and the vesicular (VGAT) GABA transporters. GAT-1 and VGAT are the first transporters to be induced as they are expressed in ES cells and parallel the neuronal differentiation while GAT4, GAT3 and GAT2 were detected after dai2 and throughout the differentiation period (***Fig.25***).

The developmental switch of GABA action from excitation to inhibition is due to reduction in intracellular chloride concentration that correlates with the induction of KCC2, the chloride potassium K+-Cl- cotransporter, and down regulation of NKCC, the sodium potassium chloride Na+K+Cl- exchanger (Ben-Ari., 2002). As expected, NKCC1 was detected in all stages studied here but gradually increased during differentiation, while KCC2 transcript could be hardly detected initially, but was upregulated in cultures after RA treatment (***Fig.25***).

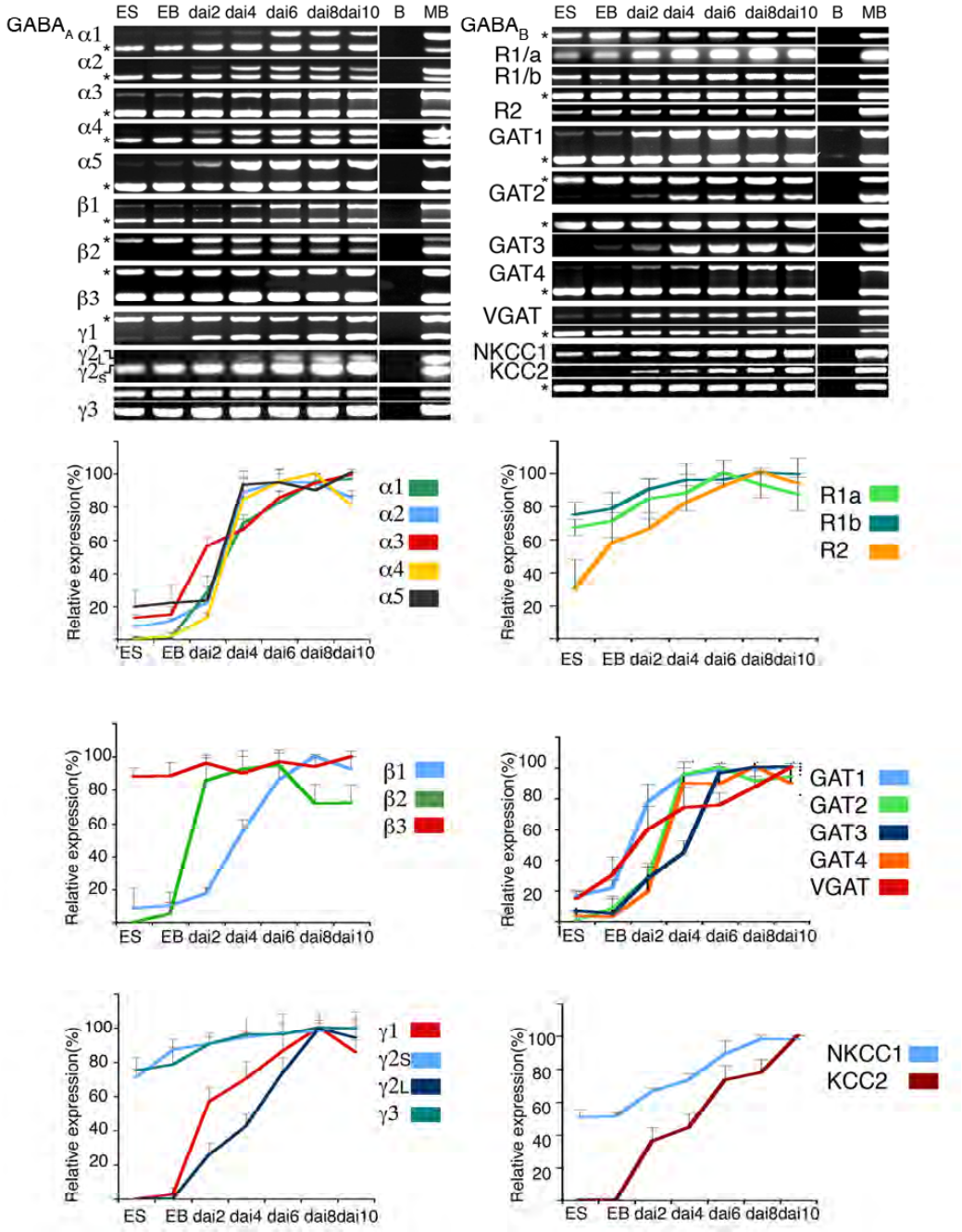


Fig.25. Temporal expression profile of GABA signaling components during neuronal differentiation of ES cells. A: Expression of $GABA_A$ and $GABA_B$ receptor subunits and GABA transporters as revealed by semi-quantitative RT-PCR. **Upper panel**: Representative gel images showing the PCR products amplified from reverse-transcribed total RNA that was isolated from from total RNA preparations of undifferentiated (**ES**) and RA-induced ES cells (**EB, dai2-dai10**), and adult brain (**MB**). Specific primer pairs derived from different exons and product size for each cDNA are listed in Table 1. β-actin (*) was co-amplified with the target genes. **Lower panel:** Relative expression levels of GABA receptor subunits and GABA transporters. Expression levels were normalized to β-actin, and represented on a grey scale as % of maximum level. **B**: Charts represent comparison of developmental expression profiles of different GABA signaling components. Expression levels were calculated from three independent experiments and normalized to β-actin and were expressed as a % of the maximum expression in each group.

2.3. *Development of GABA immunoreactivity in ES-derived neurons during in vitro differentiation*

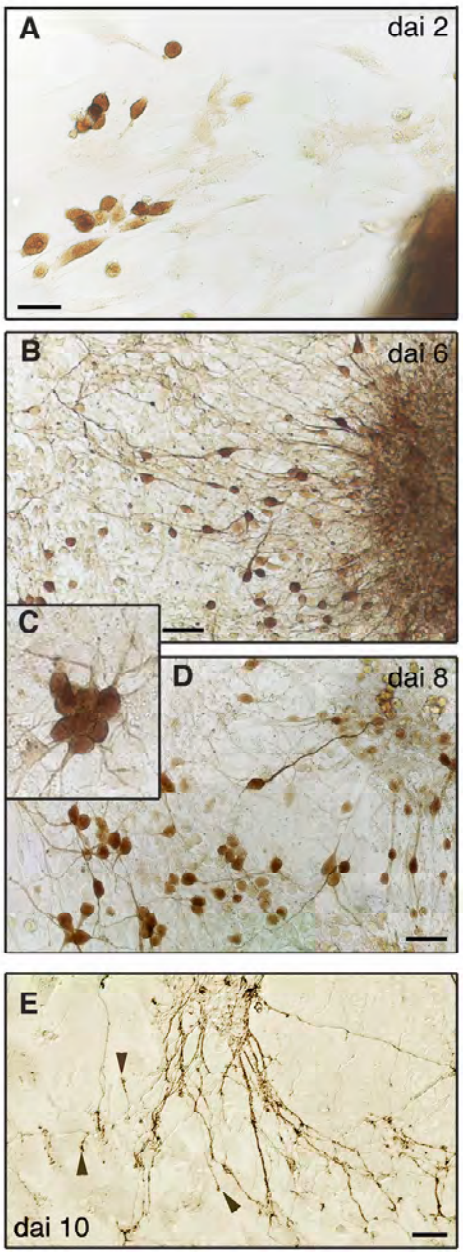


Fig.26. Immunocytochemical detection of GABA in cells at different stages of neuronal differentiation. Images were taken on second (dai2; A), sixth (dai6;B), eighth (dai8;D) and tenth (dai10;E), respectively. Arrows show GABA positive growth cones. Scale bars:20μm in A, 40μm in B,D and E.

GABA immunoreactivity was examined during different stages of *in vitro* neuronal differentiation of ES cells. At the earliest time point used, two days after plating, cultures consisted mostly of flat, fibroblast-like cells and few long neurites protruding from EBs that were not immunopositive for GABA. However, in some areas we detected single cells, with migratory appearance, displaying strong staining in both perikaria and short, branched processes (***Fig.26A***). In the following days, cells start to migrate in large numbers from EBs, GABA immunoreactivity increased drastically in both cell bodies and neurites. By day 6, most of immunopositive cells, ranging from bipolar to multipolar neurons, formed long dissimilar neurite-like processes radiating from EBs, also $GABA^+$ (***Fig.26B***), while some of the young neurons formed tight clusters containing 10-30 cells (***Fig.26D***) or fascicles (not shown).

In cultures fixed 8 days after induction perod we could not detect any GABA immunoreactivity in the EBs. The labeled neurons with morphology of migrating cells had longer neurities and became progresivelly spread out in the culture (***Fig.26D***). By day 10 after RA induction the morphology of GABA-containing neurons changed dramatically by apearance of long, bundled processes (***Fig.26E***). Growth cones were also strongly labeled for GABA: some of them had a large number of filopodia, others had multiple long extensions arising from a long neurites (arrows). Flat cells with appearance of fibroblasts or glia in our cultures were not imunoreactive for GABA.

3. GABA SIGNALING IN THE DEVELOPING MOUSE LENS

Earlier work from this laboratory demonstrated, by using GAD *in situ* hybridization, that mRNAs for both GAD65 and GAD67 are present in the elongating lens fiber cells of mid-gestation mouse embryo (Katarova et al., 2000). Here we examined the temporal and spatial expression of different GAD forms, GABA receptor subunits and GABA transporters (vesicular VGAT and membrane GATs) in the developing mouse lens and in primary lens epithelial cultures (LEC) and present evidence about the functionality of GABA signaling in the developing mouse lens.

3.1. *GAD mRNAs are sequentially expressed in the developing lens*

The temporal expression pattern of different mRNAs encoded by the two GAD genes was detemined during lens development by 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. Embryonic stage E14.5, when the lens is composed of epithelium and predominantly primary lens fibers, was chosen as a starting point due to technical limitations.

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 (***Fig.27***). The embryonic GAD transcripts encoded by the GAD67 gene (EGADs) 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 postnatally. On the contrary, GAD67 was expressed at a constant low level until birth (P0), with a slight peak at E16.5, and then gradually increased, peaking postnatally at P14-P30. Similar to EGADs, GAD65 mRNA level was already high at the primary fiber stages (E14.5) peaked at E15.5 then decreased with a sharp decline from E17.5 and was undetectable at P14 and beyond (***Fig.27***).

We also examined for the presence of Dlx2 and Dlx5, two transcription factors that

overlap with expression domains of GAD (Katarova et al., 2000) and considered to be the upstream regulators of GAD. Our semi-quantitative RT-PCR showed that similarly to the forebrain, Dlx2 expression in the developing lens was induced before Dlx5. As shown in Fig.23, Dlx2 mRNA levels were high and remained almost unchanged throughout E14.5, E15.5 and E16.5, after which Dlx2 expression declined and was undetectable in P7 and older lenses. 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 down - regulated and was not detected beyond P30 (***Fig.27***).

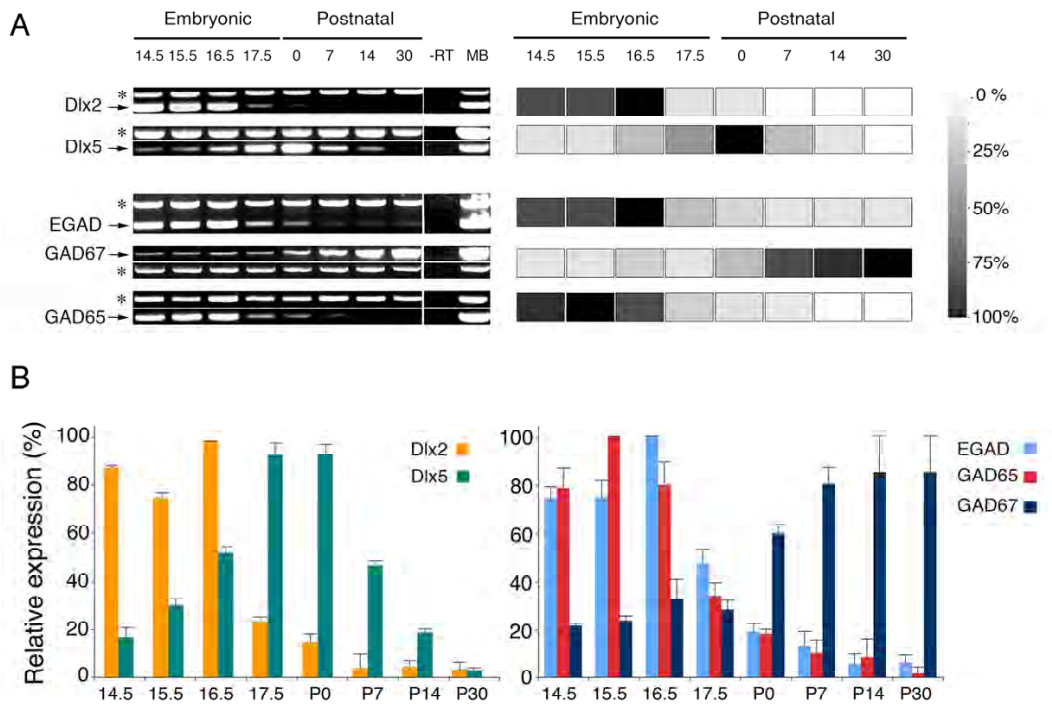


Fig.27. Temporal expression patterns of Dlx2, Dlx5 and GAD gene family in developing mouse lens as revealed by semi-quantitative RT-PCR. A: Representative RT-PCR amplifications of Dlx2 and Dlx5 and different GAD (EGAD, GAD67 and GAD65) transcripts using cDNA synthesized from total RNA preparations of E14.5-P30 lenses and adult brain (MB). Expression levels were normalized to β-actin (*), and represented on a grey scale as % of max level. B: 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%).

3.2. *$GABA_A$ and $GABA_B$ receptor subunits and GABA transporters are abundantly expressed in the developing lens*

We determined the temporal expression profile of mRNAs encoding different $GABA_{A,}$ subunits, the two $GABA_B$ receptor subunits, the vesicular and membrane GABA transporters (VGAT and GAT1-4, respectively) during lens development (stages E14.5-P30) by semi-quantitative RT-PCR (***Fig.28***). Out of the six α subunits (α_{1-6}) amplified using specific oligo-pairs, we failed to detect α_5 and α_6 at any stage during lens development (not shown). α_2 and α_3 subunits were detected at the earliest stages (E14.5-E15.5) and were abundantly expressed until P0, thereafter α_2 was present at low levels. Subunits α_1 and α_4 were virtually undetectable at E14.5-E15.5. While α_1 expression level rose continuously from E16.5, α_4 elevated steeply at E16.5 and remained at constant high-level expression thereafter.

Of the three known β subunits, β_1 displayed only slightly over-background expression throughout all studied stages with the exception of P0 when it was up-regulated to a small degree. This unusual profile was consistent in multiple experiments (data not shown) and may reflect a special need for this subunit at birth. β_3 was by far the most abundant subunit at embryonic stages E14.5-E17.5, but was dramatically reduced after P0. The $GABA_AR$ β_2 subunit showed low expression at earlier (E14.5-E15.5) and late (P14-P30) stages and a slightly higher expression at E16.5-P7 (***Fig.28***).

All three $GABA_AR$ γ subunits and the δ subunit (a substitute for γ) were expressed from the earliest studied stages. γ_1 is predominantly prenatal and almost undetectable beyond P0 with the peculiar exception of P30, while γ2, γ3 and δ were present at all studied stages. Interestingly, γ_3 subunit is by far the most abundant of all γ subunits during all stages of lens development, which contrasts its highly restricted expression in the brain (Laurie et al., 1992). We failed to detect the retina-specific ρ1

and ρ2 subunits (not shown), which demonstrates that all RT-PCR reactions are strictly lens-specific.

The functional $GABA_B$ receptor is an obligatory heterodimer comprised of two subunits: $GABA_BR1$ harboring the GABA binding site and $GABA_BR2$, responsible for the dimer formation (Bowery and Brown, 1997; Kaupmann et al., 1997). While both R1 and R2 were expressed in the developing lens, they displayed strikingly different expression profiles (***Fig.28***). $GABA_BR1$ showed strong and uniform expression throughout all stages, whereas $GABA_BR2$ subunit was expressed at moderate levels at E15.5-E17.5 peaking at E16.5 and was hardly detectable at earlier and later stages.

Transcripts for all GATs and VGAT were abundant and showed distinct profiles during different stages of lens development (***Fig.28***). The predominantly neuronal-specific GAT1 showed a much higher expression around birth (E17.5-P0) than at earlier or later stages. GAT2 and GAT3 found in both neurons and glia were hardly detectable at E14.5-E15.5, but were upregulated at the later stages. The GAT4 was nearly uniformly expressed throughout all stages being slightly increased at E15.5-E16.5. VGAT was much more abundant before birth, showing a transient peak at E17.5, but was greatly reduced after birth. These patterns clearly followed different stages of fiber cell differentiation.

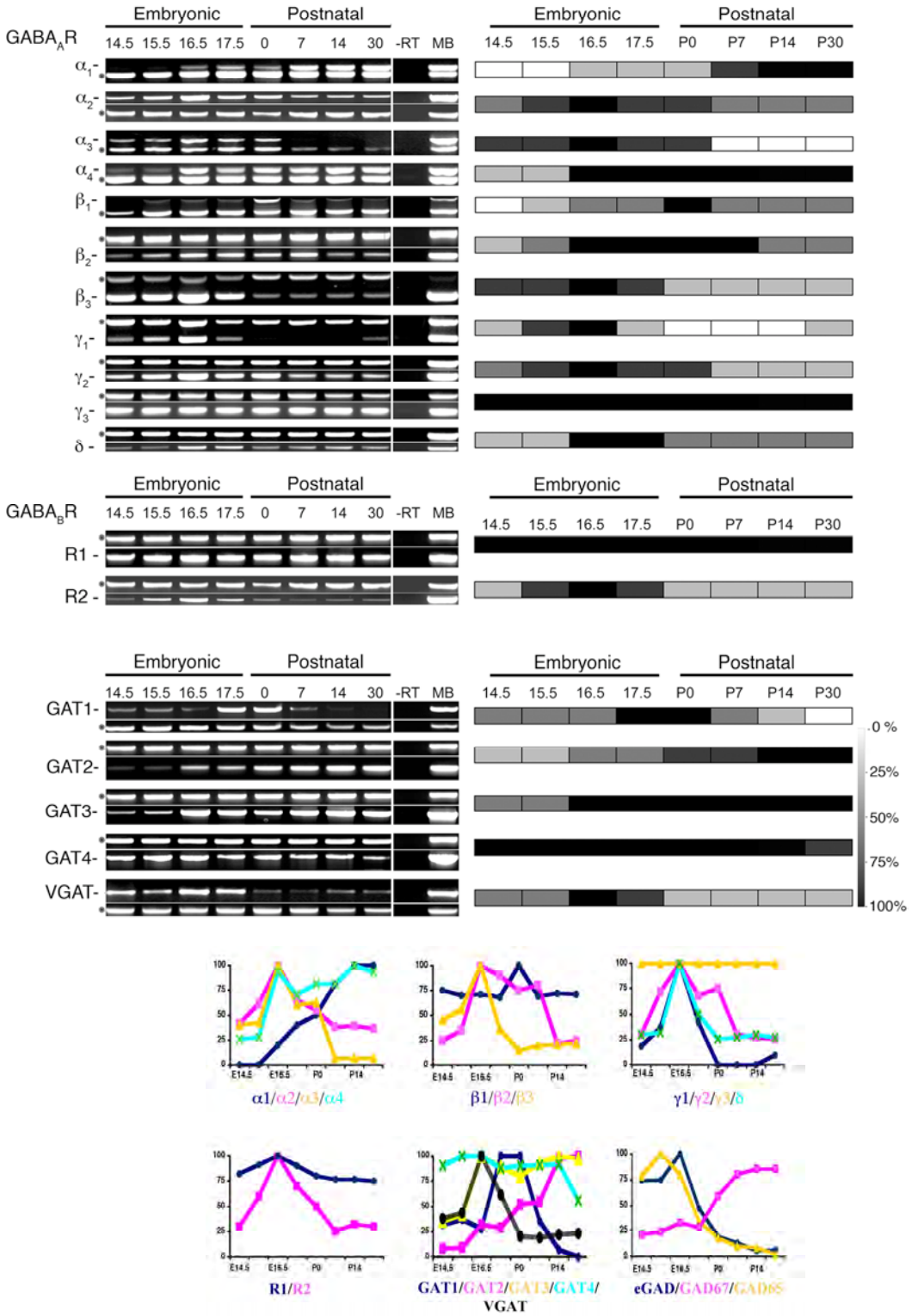


Fig.28. Temporal expression profile of GABA signaling components in the developing mouse lens. A: Expression of $GABA_A$ and $GABA_B$ receptor subunits and GABA transporters as revealed by semi-quantitative RT-PCR. **Left panel**: Representative gel images showing the PCR products amplified from reverse-transcribed total RNA that was isolated from embryonic (E14.5-17.5) and postnatal (0, 7, 14 and 30 days) lenses and adult brain (MB). Specific primer pairs derived from different exons and product size for each cDNA are listed in Table 1. β-actin (*) was co-amplified with the target genes. **Right panel:** Relative expression levels of GABA receptor subunits and GABA transporters, as assayed by the fluorescence intensity of the PCR products (left panel). Densitometry analysis was performed using IMAGE (NIH, v. 1. 34.). Expression levels were normalized to β-actin, and represented on a grey scale as % of maximum level. **B**: Charts represent comparison of developmental expression profiles of different GABA signaling components. Expression levels were calculated from three independent experiments and normalized to β-actin and were expressed as a % of the maximum expression in each group.

3.3. *Spearman correlation identifies similar expression profiles of different GABA signaling components during lens development*

The striking similarities between expression patterns of different GABAergic components prompted their grouping according to developmental stage (embryonic, fetal, neonatal and postnatal; ***Fig.29A***). During embryonic stages mostly GAD65 and the embryonic GAD trancripts are expressed and this is paralleled by predominant expression of VGAT and the membrane GAT4. The prevalent configuration of the $GABA_AR$ at this stage is $\alpha_{2,3,4}$ /β_3/$\gamma_{2,3}$. This receptor type predominates in the spinal cord at early embryonic stages (Ma et al., 1993) which points to the existence of common features of development of the GABA signaling pathway. In the fetal lens most of the GABA signaling components are greatly up-regulated in correlation with the increased speed of generation of lens fibers during this period (Kaufman, 1993). GAD65 and EGAD continued to predominate, paralleled at the beginning by VGAT and GAT4, and later by GAT3 and GAT1 (***Fig.29A***). The predicted $GABA_AR$ pentamer during this period is $\alpha_{2,3,4}$/$\beta_{2/3}$/$\gamma_{1,2,3}$ with all these subunits highly expressed.

Around birth (neonatal stage), $GABA_AR$ β1 and GAT1 were selectively upregulated and the embryonic/fetal components were supressed.

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_1, γ_2, δ, $GABA_BR2$ subunits, up-regulation of GAT1,2,3 and the $GABA_AR$ α_1 subunit, which is predominantly synaptic in brain.

These findings gained further support from the Spearman correlation analysis (Kotlyar et al., 2002; ***Fig.29: B1-B4***), which clearly showed high expression

correlation between the predominantly prenatal GAD65 and EGAD (r_s=0.94), GAD65-$GABA_AR\alpha_2$ (r_s=0.80), $GABA_AR\alpha_2$-$GABA_AR\gamma_2$ (r_s=0.86) and GAD65-VGAT (r_s=0.67). GAD67 expression was highly correlated with that of GAT2 (r_s =0.99) and $GABA_AR$ α_1 (r_s=0.96; Fig. 29B5, B6), all expressed postnatally.

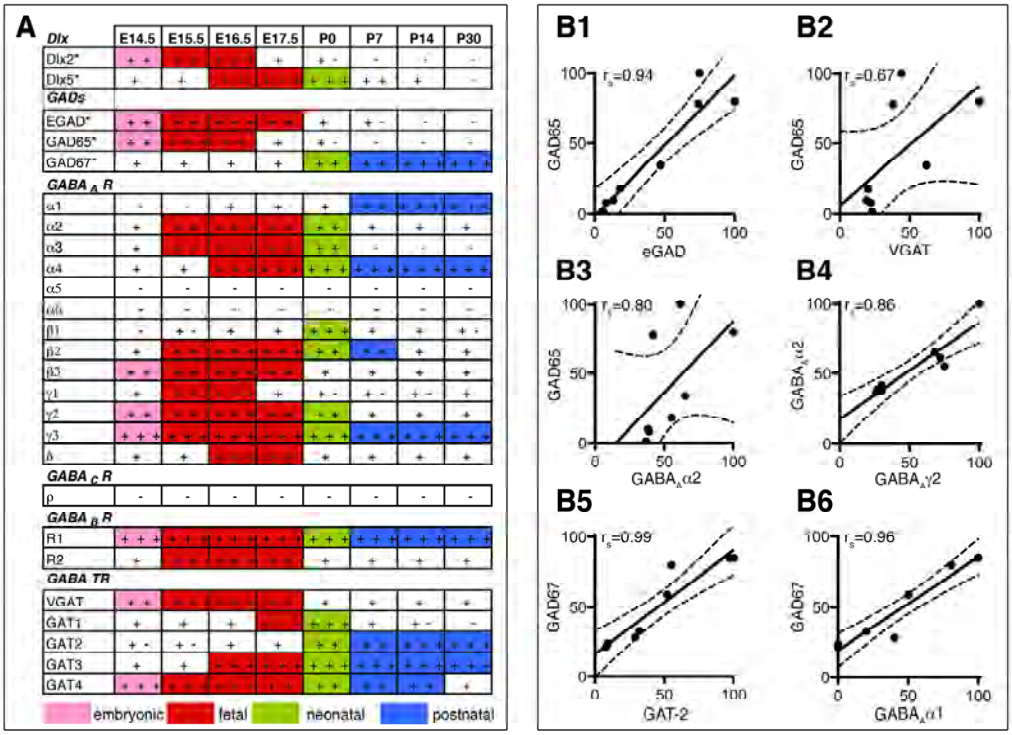


Fig.29. **Developmental stage-specific expression profiles and coordinated expression of genes of GABA signaling components in the developing mouse lens. A:** Schematic presentation of temporal expression profiles based on RT-PCR data in Fig.L1A and L2A Relative expression level of each gene at different developmental stages (E14.5-P30) indicated as follows: +++: high; ++: medium; +: low; -: no expression. Dvelopmental stages: embryonic ≤ E14.5; fetal: E15.5-E17.5; P0: neonatal; postnatal: P7-P30. **B:** Coordinated expression of GAD, GABA receptor subunits and transporters: eGAD vs. GAD65 (**B1**), VGAT vs. GAD65 (**B2**), GABAAα2 vs. GAD65 (**B3**), GABAAγ2 vs. GABAAα2 (**B4**), GAT-2 vs. GAD67 (**B5**) and GABAAα1 vs. GAD67 (**B6**). Pair-wise correlation analysis of relative gene expression levels at different developmental stages was performed using Prism software. Spearman's rank correlation coefficient (r_s) 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.

3.4. *Temporal expression of GAD proteins and GABA in the developing lens*

We used Western blot and HPLC analysis to study the synthesis of GAD proteins and GABA content through E15.5 embryonic-P30 postnatal stages. The 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 (Katarova et al., 1990; Szabo et al., 1994). β-actin was used to monitor the amount of protein applied on the gel. P30 retina homogenate expressing high levels of

all GAD isoforms was run in parallel (***Fig.30A*: 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.30A***). EGAD (GAD25 and GAD44) showed a transient temporal expression pattern similar to that characteristic for the embryonic nervous system (Szabo et al., 1994): it was low at the earliest stages (E15.5), peaked at E17.5, gradually decreased until P14 and was undetectable beyond this stage (***Fig.30A***).

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.30B***).

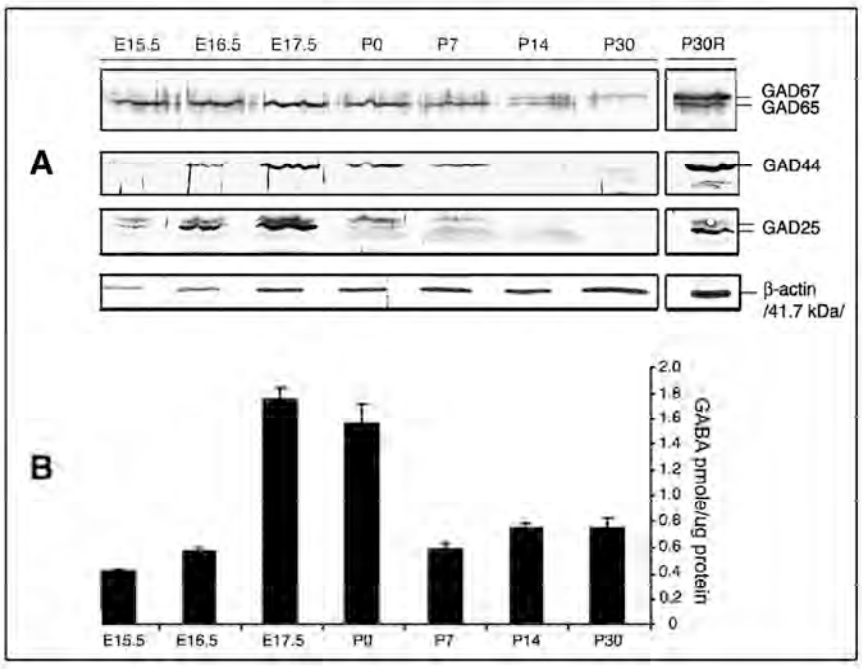


Fig.30. GAD proteins and GABA content in the developing mouse lens at stages E15-P30. **A**: Western blot analysis of GAD forms in the eye lens: protein homogenates from lenses isolated from the different stage embryonic lenses next to P30 retina (P30R) were run on 10% SDS-PAGE, blotted and stained with an anti-GAD rabbit serum #6799. β-actin was stained as control. **B**: Analysis of GABA content in the developing mouse lens: GABA concentration was measured in E15.5-P30 lens homogenates by HPLC and expressed in pmole/μg protein. Bars represent means of tree measurements ± SEM. GABA measurements by HPLC were done in collaboration with Mária Baranyi.

3.5. *GAD protein expression in the developing lens as revealed by immunohistochemistry: spatial segregation of different GAD forms*

The spatial pattern of GAD65 expression in embryonic lenses was revealed by immunostaining with the GAD65-specific N-65 antibody of E9.5-E17.5 eyes (***Fig.31*: *a,b,c,d,e***). Strong GAD65 staining was first visible in the thickened surface ectoderm of the lens placode (***Fig.31a***) and persisted through the lens pit (E10.5; ***Fig.31b***) and lens vesicle (not shown). At E12.5, strong labeling was detected in both, prospective lens epithelium and elongating cells of posterior wall - prospective primary lens fibers (***Fig.31c***). The core lens fibers were still prominently stained at E14.5 (***Fig.31d***), thereafter the intensity was reduced (E17.5; ***Fig.31e***). Faint GAD65 expression was still detected in P0 and P7 lens nucleus, but disappeared at later stages (not shown).

To detect the GAD67 gene-encoded GAD isoforms we used an anti-GAD antibody (serum #6799), which was raised in our laboratory against recombinant mouse GAD67, but also recognizes the embryonic GAD25 and GAD44. This antibody recognizes to a lesser extent GAD65 on Western blots, but not on tissue sections under the conditions used in this study. 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) mRNA in embryonic and predominantly that of GAD67 in the postnatal lens. As shown on ***Fig.31a'***, GAD67 staining was already detected in the lens placode, an area of a thickened surface ectoderm and the underlying optic vesicle at E9.5. At E10.5, uniform immunolabeling was observed in the lens cup (***Fig.31b'***). 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.31c'***).

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.31e'***). This pattern

was preserved until birth. In postnatal lenses, a gradual change could be followed from moderately strong intensity to complete absence of staining (P30) of the lens core concomitant with increase in immunolabeling of elongating secondary fibers at the equatorial region (EQ in P14, P30).

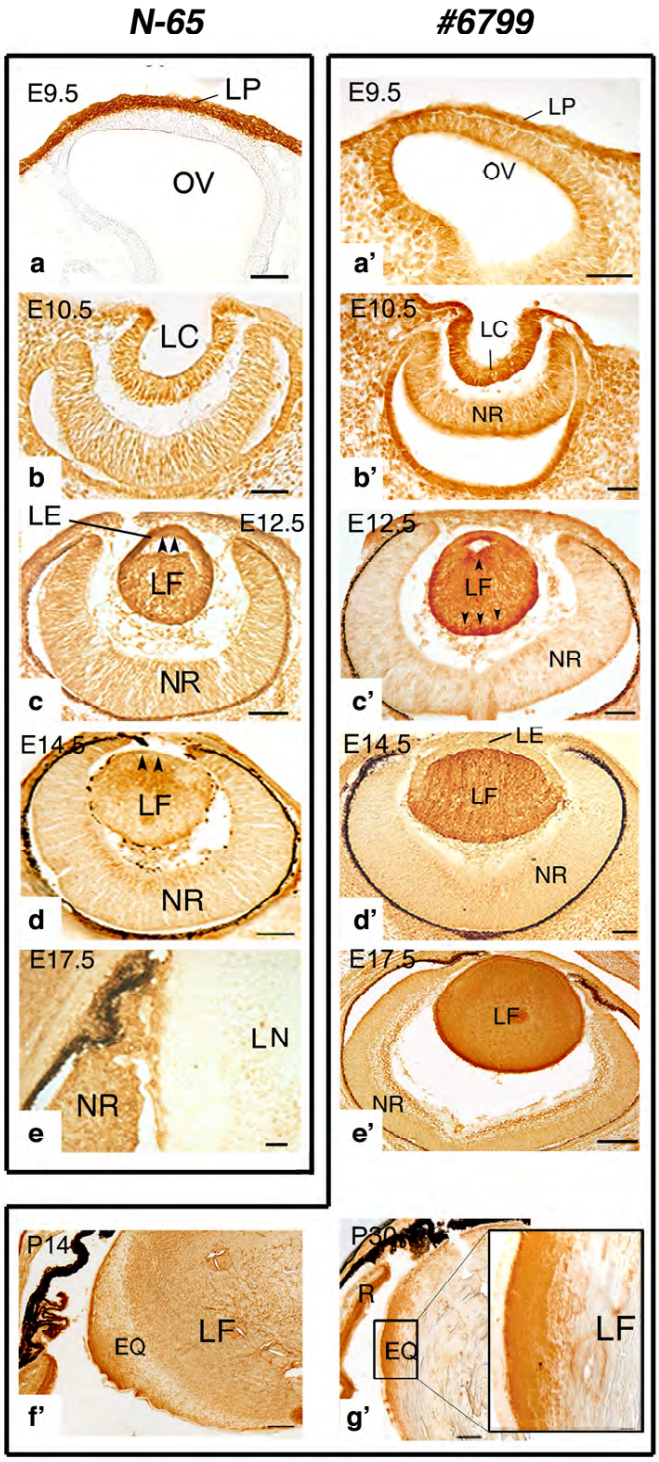


Fig. 31. Expression pattern of GAD proteins in the developing mouse lens.
a-e: coronal sections from different stage embryos stained with anti-GAD65 antibody N-65. GAD65 protein was localized initially to the lens placode, lens vesicle and later to lens primary fiber cells. Arrowheads point to lens epithelium **a'-g'**; Coronal sections from E9.5-P30 eyes were stained with anti-GAD serum #6799. Not that 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. **LC**- lens cup**; LF**- lens fibers**; LP**- lens placode; **NR**- neural retina; **OV**- optic vesicle.
EQ-equator; Arrowheads in point to the high accumulation of immunostaining product at the anterior/posterior lens poles. Scale bars: a-e, a'-e', 100μm;f'-g'. 50 μm.

3.6. GABA receptors and transporters exhibit spatially restricted localization in the newborn mouse lens

The cellular distribution of vesicular and membrane GABA transporters and the two main GABA receptor subtypes was studied by immunohistochemistry using specific antibodies on tissue sections from eyes at different developmental stages. The ionotropic $GABA_AR$ and metabotropic $GABA_BR$ were detected with antibodies specific for $GABA_A\beta3$ and $GABA_BR2$, the subunits indispensable for functional assembly of the receptors (see Introduction Chapter). Here we describe the staining patterns at neonatal stage (P0) (***Fig.32***). Staining patterns were quite similar between E16-P0. In neonatal lens labeling with all antibodies was strong in the equatorial secondary fibers and overlaying lens epithelium and extremely weak in the lens nucleus, composed of primary lens fibers. GAT1 spanned a larger expression domain of the lens cortex (***Fig.32b***), in accordance with the elevated expression of GAT1 mRNA at this stage. VGAT and the GABAR showed a strongly segregated expression in the apical/basal membranes of epithelial cells and the closely positioned cilliary body implying the existence of intensive $GABA_{A(B)}$- mediated signaling between these domains (***Fig.32: a,d,e,e'***).

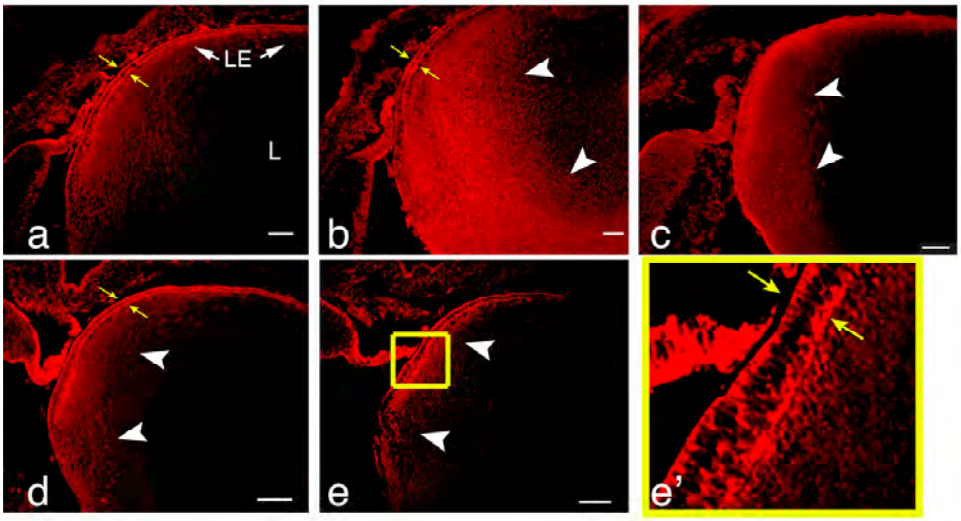


Fig. 32. Cellular localization of the GABA transporters and receptors in the newborn mouse lens: images of coronal cryosections of P0 mouse lens stained with antibodies specific for VGAT (**a**), GAT1 (**b**), GAT3 (**c**), $GABA_A\beta3$ (**d**) and $GABA_BR2$ (**e,e'**). Immunoreactivity for all antibodies is predominantly localized to the apical/basal membranes of lens epithelial cells(,yellow arrows on a-d,e') and to the equatorial fibers. Note that GAT1 staining shows more expanded expression in the cortical region containing secondary fibers compared with VGAT, GAT3, $GABA_A\beta3$ and $GABA_BR2$ (arrowheads). Abbrevations: L-lens, LE-lens epithelial cells. Scale bars: 50 μm .

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

In the newborn (P0) mouse lens GABA is predominantly localized in the lens epithelium and fiber cells with the strongest labeling found at the equatorial region and the forming sutures (***Fig.33I: a,c,e***), a pattern entirely consistent with the expression of the two GAD isoforms and the GABA signaling components (see Figs. 31,32). 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, but became strong again in external, still nucleated primary fibers (***Fig.33I: a-c***). The nuclei followed a concomitant change in shape and chromatin compaction followed by nuclear membrane disintegration (***Fig.33I: e-e'***). 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.33II: A-D***). In the intact lens only epithelial and equatorial fiber cells were loaded with fluo-4, since they have sufficient esterase activity (Bassnett et al., 1994). Applied GABA evoked an increase in fluorescence intensity mostly in cells of the equatorial region that are undergoing transition from epithelial to fiber cells (***Fig.33II: A-C***). 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.

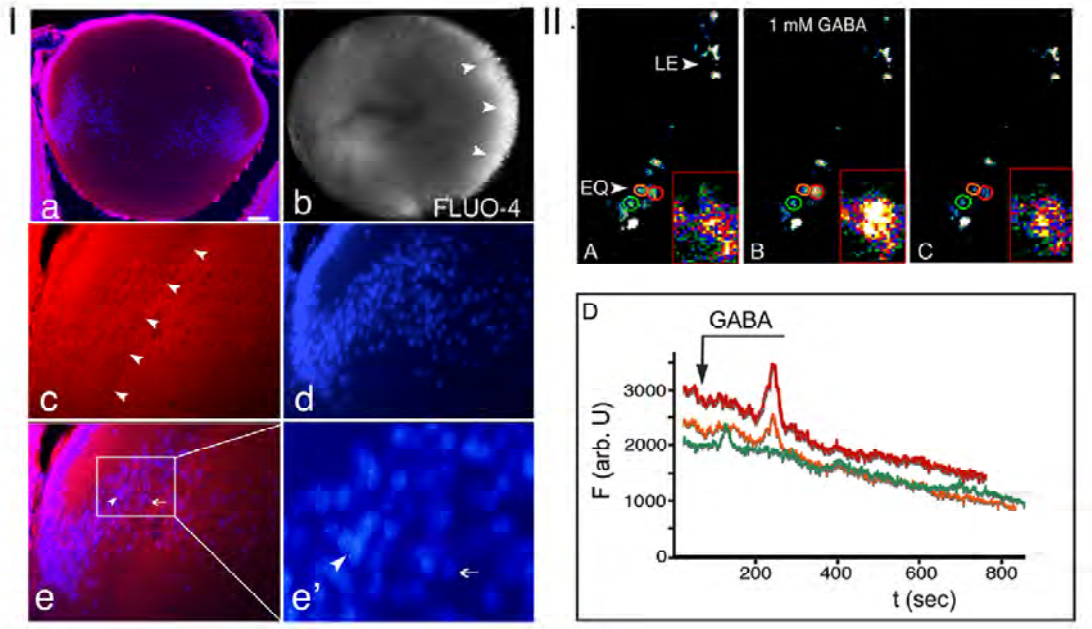


Fig. 33. GABA expression and function in modulation of $[Ca^{2+}]_i$ in the neonatal lens. **I:** Staining with GABA antibody of the neonatal mouse lens (**a, c, e**). Nuclei were visualized by DAPI staining (**a, d, e, e'**). GABA immunoreactivity is visible predominantly in equatorial secondary fibers possessing intact nuclei (arrowheads in **e, e'**) and in primary fibers with nuclei in the process of disintegration (arrows in **e, e'**). The border between primary and secondary fibers is well demarcated by strong GABA staining as is the equatorial lens region containing the newly generated secondary fibers (arrowheads in **c**). 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-e,

To overcome these difficulties, we performed Ca^{2+} imaging in primary LEC culture, an *in vitro* system successfully used before for similar studies (Churchill et al., 2001). To this end first we established and characterized the primary LEC culture system, then we tested whether the LEC cells express in vitro the molecular components of GABA signaling.

3.8. *Characterization of LEC cultures*

Primary LEC cultures were derived from newborn mouse lenses according to a modified protocol and were used for further studies (Fig.28). P0 was chosen as a stage with high expression levels of GABA signaling components as well as ample supply of easy-to-dissect lens epithelial and young fiber cells. As shown in ***Fig.34***, after two weeks primary LEC cultures were comprised of both undifferentiated epithelial cells that can be spread or form epithelial monolayers (***Fig.34: e,f)*** and randomly distributed lentoids- multilayered cell aggregates that appear to bulge from the surrounding epithelial cells (***Fig.34: d,e***). On the basis of morphological criteria and expression profiling they are considered an equivalent of elongating newly-formed fiber cells (Menko et al., 1984; TenBroek et al., 1994; Frenzel and Johnson, 1996; Wagner and Takemoto., 2001).

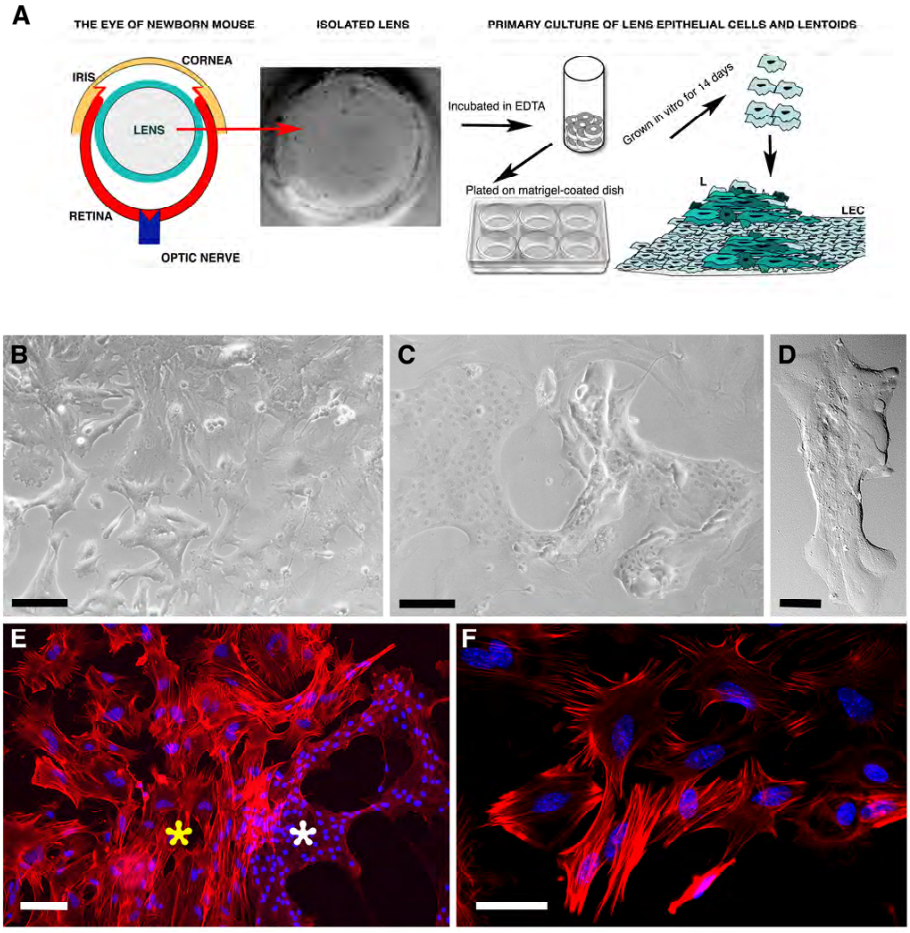


Fig.34. LEC culture established from newborn mouse eye. A: Scheme for lens cell isolation procedure and establishing of LEC culture. Following removal of retina and vitreous body from eyes of newborn (P0) mice, lenses were incubated in EDTA solution, centrifuged and plated in M199 medium on matrigel-coated coverslips**. B-F**. Images of LEC after seven (B) or fourteen (C-F) days in vitro by which time culture comprised of epithelial monolayers (E,F) and spontaneously differentiated fiber cells in lentoids (C,D,E) . Cells were visualized by phase contrast (B, C), DIC (D) or by immunofluorescence phalloidin staining for filamentous actin (E, F). Stars in E indicate the area of epithelial cell monolayer (yellow) and lentoids (white). The cell nuclei were counterstained with DAPI (blue). Scale bars: 100μm (B, C, E), 50μm (D, F).

First, we examined the cultures for the expression of cellular markers and lens fiber specific proteins. The molecular chaperone αA-crystallin, one of the most abundant proteins of the lens epithelium and fibers (Piatigorsky et al., 1989) and the small αB-crystallin, more abundant in fiber cells were both detected in lentoids and epithelial cells (***Fig.35: A,B,D***). Lentoids, but not single epithelial cells stained strongly for N-cadherin (***Fig.35C***), while E-cadherin only stained the membranes of epithelial monolayers, but not lentoids (***Fig.35: E,G***).

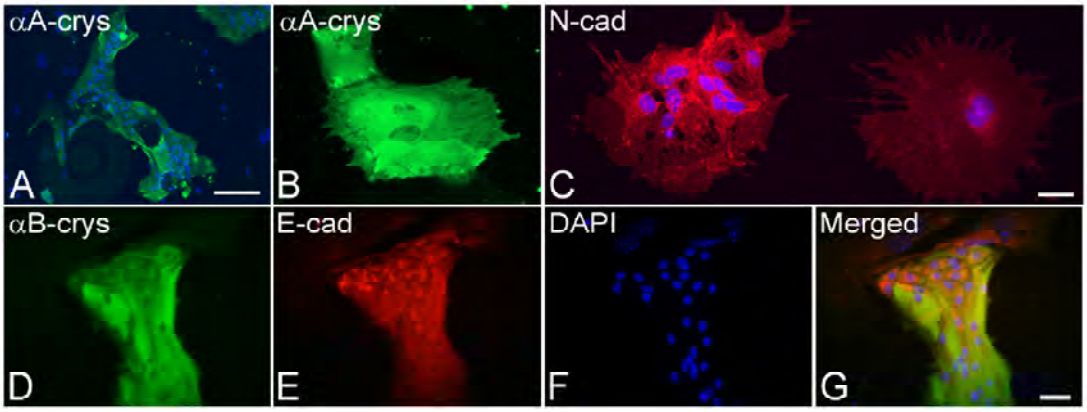


Fig.35. Characterization of mouse lens epithelial primary cultures Immunofluorescent staining of primary LECs with antibodies to lens specific αA- and αB-crystallin, E- and N-cadherin. *A, B, D.* As the cells began to differentiate and form lentoids and as the lentoids began to become more highly differentiated, 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: 100μm in A, 50 μm in B-C, 20 μm in D-G.

3.9. *Lens epithelial cells and lentoids grown in vitro express key GABA signaling components and contain GABA*

Similar to intact lenses, cells in primary lens epithelial cultures prepared from P0 lenses expressed GAD65 and GAD67 forms that co-localized with the vesicular and membrane GABA transporters, respectively (***Fig.36***). In general, lentoids showed the strongest expression, while epithelial monolayers were less prominently stained. Some $GAT1^+$ cells in LEC cultures did not express GAD (***Fig.36: E,G,H***), which suggests that GAT1 in these cells operates as GABA uptake only, similar to CNS glia involved in the GABA clearing from the synaptic cleft.

Moreover, strong labeling of $GABA_A\beta3$ and $GABA_BR2$ was detected over the cell borders of the lens epithelial monolayers and lentoids (***Fig.37***). The staining had a punctate appearance and was greatly enriched near cellular membranes (***Fig.37: A' B'***). Single cells showed little or no staining (data not shown). In conclusion, epithelial sheets and lentoids expressed all GABA signaling components required for building up a functional pathway.

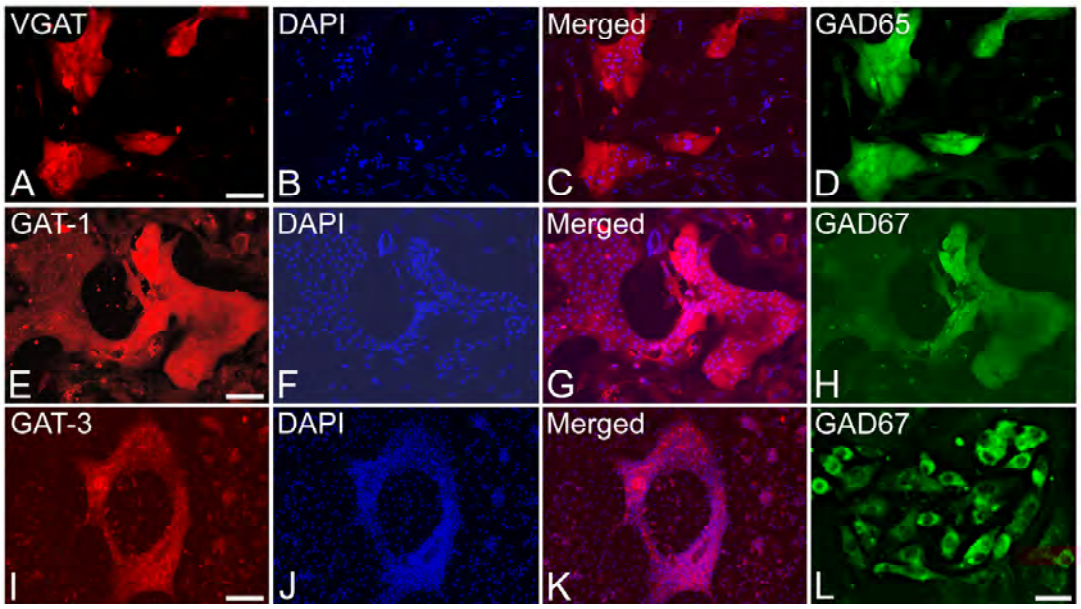


Fig.36. Expression of GAD and GABA transporters in primary cultures of mouse lens epithelial cells (LEC). *A-D.* VGAT entirely co-localizes with GAD65; staining of the same preparation with the polyclonal anti-VGAT serum and monoclonal anti-GAD65 antibody GAD-6. *E-H.* Co-localization of GAT1 and GAD67 The membrane localization of GAT1 is more evident in the less prominently stained epithelial monolayer than the strongly positive lentoid *I-K.* Staining with anti-GAT3 antibody. *L.* GAD67 is also found in small cells outside lentoids and monolayers that are positive for αA-crystallin (not shown). Scale bar:100 μm in A-K, 50μm in L.

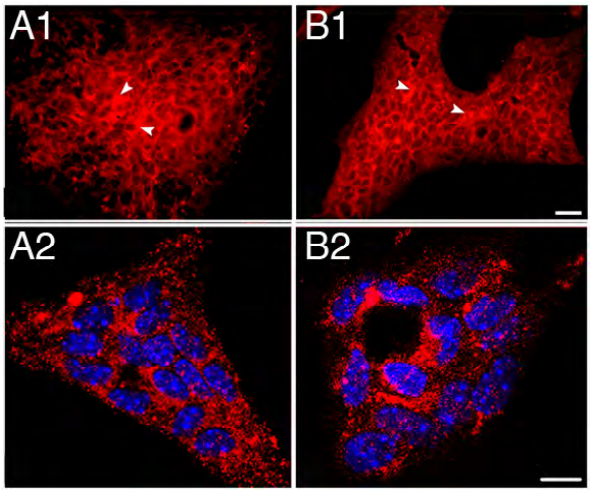


Fig. 37. Expression GABA transporters receptors and GABA in primary cultures of mouse lens epithelial cells (LEC). $GABA_A$ and $GABA_B$ receptors are enriched in multicellular lentoids. LEC cultures were stained with rabbit anti-$GABA_A\beta3$ (**A**) or anti-$GABA_BR2$ (**B**) antibodies and visualized by epifluorescence (**A1,B1**) or laser scanning microscopy (**A2,B2**). Nuclei were revealed by DAPI staining (**A2,B2**). The staining is greatly enriched near cell borders and at the surface of lentoids (arrowheads in A, B). Scale bars, 20 μm in A, B, 10 μm in A1,B1.

Finnaly, GABA synthesis and accumulation in primary LEC was verified by staining with a specific antibody (***Fig.38***). Lentoid structures showed much higher accumulation of GABA compared to adjacent LEC monolayers (***Fig.38: a, a'***), while single cells remained unstained (***Fig.38: c, c'***). This expression gradient was typically found in the vicinity of all lentoids and was accompanied by a gradual change in

nuclear shape and chromatin condensation reminiscent of the inwardly directed progressive nuclear loss in maturing fibers of intact lenses (***Fig.38: b, b'***; see also Fig. 33). Thus, primary LEC cultures provide a useful and matching model to study lens fiber differentiation.

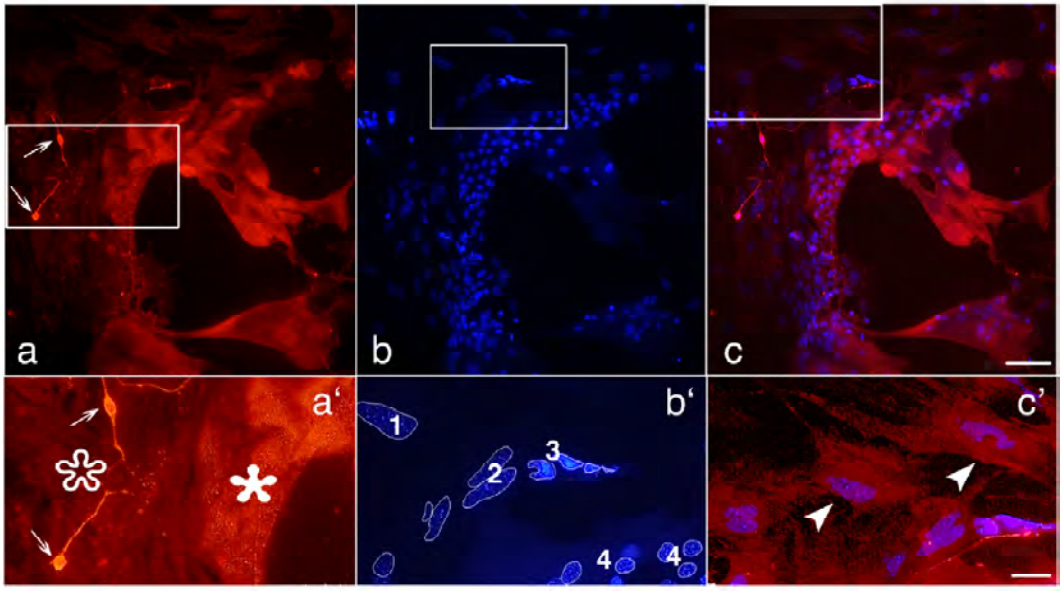


Fig.38. GABA is produced in LEC cultures. Primary LEC cultures derived from P0 mouse lenses were stained with a rabbit antibody against GABA (**a, a'; c, c'**). Nuclei were stained with DAPI (**b, b'**). Boxed regions in a, b, and c are enlarged in a', b' and c', respectively. A large multicellular lentoid-like 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'.

3.10. *GABAR-mediated modulation of $[Ca^{2+}]_i$ levels in primary LEC and lentoids*

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 remained unlabeled (data not shown).

GABA (1mM) and $GABA_AR$ agonist muscimol (100 µM) induced a simultaneous increase of fluorescence intensity in epithelial cells or cell groups belonging to

smaller lentoids (not shown). Cell-to-cell propagation of the signal, sometimes reaching out to adjacent lentoids was clearly observed and it was rarely followed by oscillations. The muscimol-evoked $[Ca^{2+}]_i$ rise was almost reversibly 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.39***). Lower number of cells responded to GABA (2.45±0.42, n = 20) than to muscimol (4.2±1.01, n=10), but the amplitude of their responses was almost identical (48.06±9.26 % and 46.5±5.49 %, respectively; ***Fig.39: C1,C2***). However, this difference may be incorrectly estimated due to difficulties in defining the contours (and ultimately the exact numbers) of reacting cells within the lentoids.

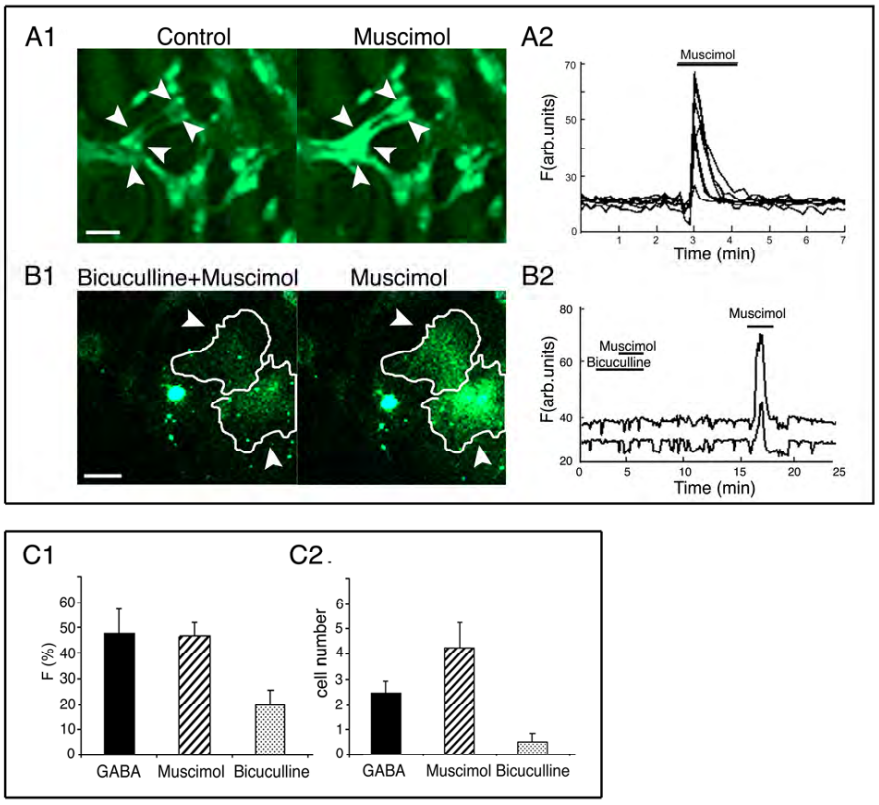


Fig.39. $GABA_A$ receptor activation induces rise in $[Ca^{2+}]_i$ in lens epithelial cultures. *A1, A2, B1, B2.* Representative recordings of $GABA_A$ receptor-evoked changes in fluorescence of Fluo4/AM-loaded primary, lens cells (arrows in A1, B1). Cultures were initially perfused with AHH buffer to obtain a steady baseline (Control) followed by 100 μM muscimol (Muscimol; A1, A2). Alternatively, following 20μM bicuculline, 100 μM muscimol and 20 μM bicuculline were co-administered (Bicuculline+Muscimol) and then 100 μM muscimol (Muscimol) alone (B1, B2). A1, B1 confocal images were taken at different time points of the recording shown in panels A2, B2. Note that the muscimol-evoked fluorescence rise was completely blocked by bicuculline (B1, B2). *C1, C2.* Bar charts illustrating the average number of cells (C1) and the amplitude change (C2) responding to GABA, muscimol alone and muscimol in the presence of bicuculline. F% represents the change in fluorescence intensity relative to the basal fluorescence. Data represent the mean ± S.D. ($p< 0.05$). Scale bar: 50 μm.

$GABA_BR$ agonist baclofen (20 μM) induced calcium transients in LEC, which differ in amplitude and kinetics between different cells and sometimes within a single cell after repeated drug applications. The baclofen-evoked transients in single cells had the smallest amplitude, which can be explained by the low number of receptors, their low sensitivity to baclofen or the presence of baclofen-evoked hyperpolarizing responses (not shown). Application of 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, which sustained even after complete washout of drugs (***Fig.40: C1,C2***). The response to baclofen was typically completely blocked in the presence of the $GABA_BR$ antagonist CGP55845. These observations were 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; Williams et al., 2001). In addition, initial aplication of CGP sometimes induced small-amplitude and short-term oscillations in single cells (not shown) which we could not explain at the moment. Furthermore, preliminary data indicates that baclofen-induced calcium transients are reduced, but not abolished, in the absence of extracelular calcium, suggesting that they might partially depend on influx of calcium from the extracellular space.

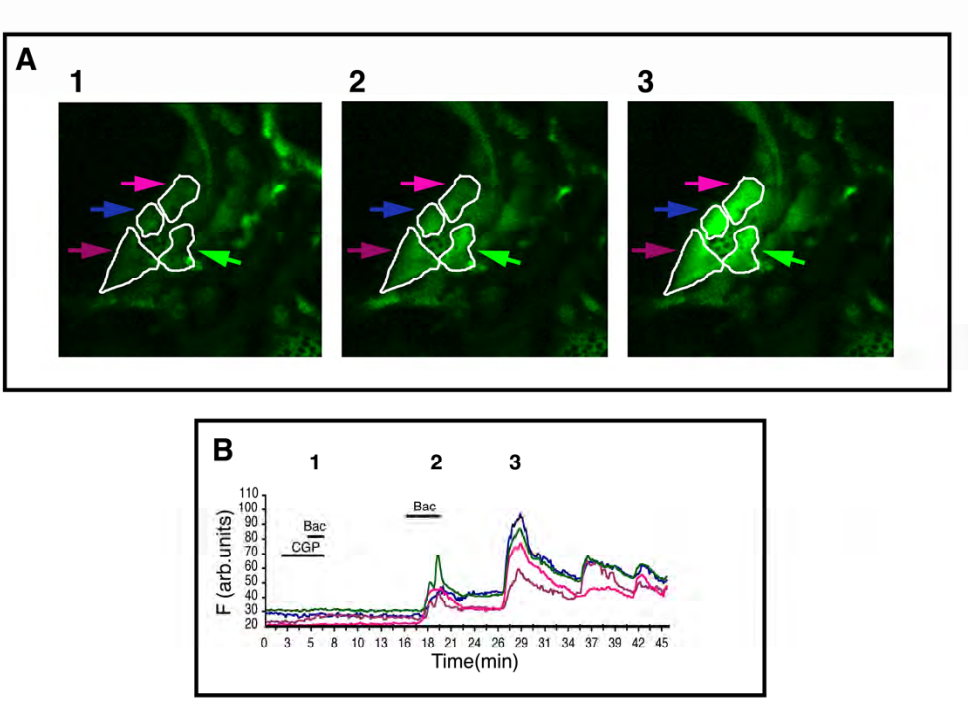


Fig.40. $GABA_B$ receptor activation induces rise in $[Ca^{2+}]_i$ in lens epithelial cultures. Cells were initially washed for 2 min with AHH buffer containing 20 μM CGP55845 (CGP) followed by 20 μM CGP+20 μM baclofen (CGP+Baclofen) and 2 min of baclofen alone (Baclofen). In some cells, mostly belonging to small lentoids, single application of baclofen induced repetitive and prolonged rices in intracellular calcium concentration.. **A**. Images (1,2,3) were taken at time point of the recordings shown in panel **B** (1,2,3, respectively). Scale bar, 50 μm.

VII DISCUSSION

1. MOUSE ES CELLS EXPRESS FUNCTIONAL GABA SIGNALING

1.1. *Structural components of GABA signaling are expressed in the mouse ES cells of line R1*

The expression of molecular components of GABA signaling in the undifferentiated ES cell was examined by RT-PCR and immunocytochemistry. We showed for the first time that the mouse ES cells, developmentally closely related to the ICM of the mouse blastocyst express all structural components needed for assembly of functional GABA signaling: GABA, GABA synthesizing enzymes (GAD65 and EGADs), membrane and vesicular GABA transporters (GAT1 and VGAT), as well as multiple $GABA_A$ (α1,α3,α4,α5,β3,γ2S,γ3,δ,ε) and $GABA_B$ (R1, R2) receptor subunits. Furthermore, the enrichment of $GABA_A\beta3$ and $GABA_BR2$ at sites of putative contacts between adjacent cellular membranes, revealed by imunofluorescent labeling, suggests that both types of GABA receptor are engaged in intensive cell-cell communications within the ES colony.

Remarkably, 11 out of 19 known to date $GABA_AR$ subunits belonging to all major subunit classes are moderately or abundantly expressed in undifferentiated mES cells providing the structural requirements for the formation of functional $GABA_A$ receptors. As previously shown, despite the high number of subunits for each subclass, there is only limited number of pentameric combinations actually exist in the CNS (Barnard et al., et al., 1998; Costa, E., 1998; Mohler, 2006). Interestingly, we detected all but two α (α2 and α6), all γ, in addition to δ and ε subunits, but only a single type of the GABA-binding β subunit (β3) in undifferentiated ES cells. The β3 gene is part of the α5-β3-γ3 gene cluster on mouse Chr. 7 (human CHR 15) implicated in Angelman's syndrome (Hommanics et al., 1997; DeLorey and Olsen,

1999) the only one that is fully turned on in ES cells, the remaining four clusters (Barnard et al., 1998) are only partially transcribed. This expression pattern may be important consequences for the $GABA_A$ receptor expression regulation and receptor properties at later developmental stages. First, it may be involved in defining a future expression paradigm for the co-ordinate regulation of the genes within a cluster, second it should allow for only a limited number of pentamers to be formed in the pluripotent ES cells. An obvious consequence of the absence of β2 subunit would be the lack of the major brain synaptic $GABA_A$ receptor type composed of 2α1-1β2-2γ2 (Mohler, 2006; Mody, 2008) and the preferential assembly of predominantly extra-synaptic receptors due to abundant expression of subunits found mostly (α5, Collinson et al., 2002) or exclusively (α4, δ and ε; Mohler, 2006; Mody, 2008) extrasynaptically. α1, which is mostly synaptic in adult brain can also localize to extrasynaptic sites when assembled with δ (Glykys et al., 2007). Notably, of the two γ2 splice variants only γ2S (short) is present, while the γ2L (long) form, which incorporates preferentially at postsynaptic sites (Meier and Grantyn, 2004) is altogether missing. The γ2S can also physically interact with $GABA_BR1$ and anchor it to the membrane (Balasubramanian et al., 2004).

However, a synaptic type $GABA_A$ receptor can still be assembled between α1-3, β3 and γ2S/γ3. Interestingly, γ3 is very restricted in adult brain (Sieghart and Sperk, 2002), but is the most abundant of all γ subunits in mouse ES cells. This observation holds true also for the developing lens (see next Chapter), and may indicate common features in the developmental programs of these systems.

In brain synaptic $GABA_AR$ mediate fast (phasic) neurotransmission, while extrasynaptic $GABA_AR$ are responsible for the slow and long-lasting "tonic" inhibition (reviewed in Mody, 2008). Extrasynaptic receptors containing δ and ε subunits are potentiated by allosteric modulators like alcohol, anaesthetics or

neurosteroids (Mohler, 2006; Mody, 2008;). Furthermore, $GABA_A$ receptor containing the ε subunit, both splice variants of which we found highly expressed in ES cells, can replace not only γ, but also α or β subunits and assemble into variable and often constitutively active receptors (Neelands et al., 1999. Bollan et al., 2008) that are readily potentiated by steroids in the absence of GABA (Bollan et al., 2008).

The metabotropic $GABA_B$ receptor was represented in ES cells by the R1a and R1b variants of $GABA_BR1$ and the $GABA_BR2$ subunit and found at the protein level in a specific vesicular pattern previously found in neural (Ritter et al., 2005) and peripheral Schwann cells (Magnaghi et al., 2004).

The enrichment of GABA receptors and GABA transporters at sites of putative contacts between adjacent cellular membranes suggests that they are engaged in intensive cell-cell communications within the ES colony.

1.2. GABA receptor of unique features act synergistically to modulate $[Ca^{2+}]_i$ in ES cells

Herein we show for the first time that in ES cells multiple $GABA_AR$ and/or $GABA_BR$ subunits assemble into functional receptors, and their activation with specific ligands triggers Ca^{2+} transients. The $GABA_AR$-mediated $[Ca^{2+}]_i$ rise in response to muscimol is clearly of dual nature due to Ca^{2+} influx through Cd^{+}-sensitive VGCC and mobilization of CPA-sensitive internal stores. Accordingly, the cellular machinery required for these responses is present in the ES and displays several specific features. First, the low level of L-type (long-lasting, high voltage-activated) channel and moderate to high expression of the N (neural), R (residual) and especially T (transient), moderate-to-low voltage activated type Ca^{2+} channels (Han and Lee, 2005; Lee et al., 2006) suggests that Ca^{2+} rise may occur at low excitation levels, but long-lasting and possibly detrimental $[Ca^2]_i$ rises are mostly avoided. Second, the

abundant expression of two out of three IP_3R (Yanagida et al., 2004,) and detectable RyR1 and RyR2 receptors suggests a greater role of the intracellular Ca^{2+} stores (see below).

The $[Ca^{2+}]_i$ increase induced by non-synaptic $GABA_A$ receptor activation in the embryonic nervous system (see Introduction) or non-neuronal cells (Kuroda et al., 1999; Xie et al., 2004) is well documented and ES cells are of no exception from the classical examples. Quite unexpectedly, however $GABA_B$ receptor activation by the highly specific agonist baclofen also induced Ca^{2+} transients, which were mostly, if not exclusively due to Ca^{2+} release from the intracellular Ca^{2+} stores (Verkhratsky, 2005) as they were insensitive to externally added Ca^{2+} or the presence of VGCC inhibitors, but could be blocked almost entirely by the ER-SR Ca-ATPase blocker CPA. This response is the opposite of the well-documented inhibitory effect of $GABA_B$ receptor on $[Ca^{2+}]_i$ rise in both mature and immature neurons and non-neuronal cells (Watanabe et al., 2002; Nicoll, 2004) and is very similar to the recently described baclofen-evoked and entirely intracellular store-dependent $[Ca^{2+}]_i$ rise in primary astroglia cultures (Meier et al., 2008).

Previous data showed that mouse ES cells or ICM express exclusively IP_3R and there is a developmental switch from IP_3R to RyR-mediated Ca^{2+} signalling (Rosemblit et al.,1999; Yanagida et al., 2004.). However, we have detected low-to-moderate expression of RyR and both RyR_1 and RyR_2 have been found in human ES cells (Goh et al., 2005).

Interestingly the $GABA_B$ receptor antagonist CGP35348 in the same experimental paradigm was similarly incapable of suppressing the Ca^{2+} rise following KCl-baclofen application pointing again at the specific features of the receptor. Thus, further experiments will be needed to determine the exact sequence of downstream

events and components involving intracellular Ca^2 stores following $GABA_B$ receptor activation in mouse ES cells.

$GABA_B$ receptors can also facilitate Ca^{2+} influx via store-operated channels (SOC, New et al., 2006) or potentiate the L-type VGCC (Carter and Mynlieff, 2004; New et al., 2006) in an external Ca^2- dependent way, although these comprise a smaller component in the overall response.

Another notable feature of the $GABA_BR$-mediated Ca^2 signalling in ES cells is the simultaneous reaction of neighbouring cells often followed by persistent oscillations that may share similarities with IP_3R-mediated response in mesenchymal stem cells (Sun et al., 2007 and ref therein).

Intercellular Ca^2 waves become important at the blastocyst stage with the increase of cell number and demand for co-ordinate cellular activity (Webb and Miller, 2003). The Ca^2 transients observed in small clones or groups of neighbouring cells on the surface of bigger clones upon activation of the $GABA_BR$ result clearly from a coordinate response most probably involving intercellular spread of Ca^{2+} and/or IP_3 through gap junctions formed by Cx43 abundantly expressed in both ES cells and ICM (Verkhratsky et al., 1998; Webb and Miller, 2003).

Surprisingly, single transients could be blocked by CGP55485 but repeated long-lasting transients were insensitive to two different $GABA_BR$ blockers (FENS, Schwirtlich et al., 2006) and may result from a constitutive activation of $GABA_B$ receptors.

It should be stressed that CGP alone could induce a moderate rise in the basal Ca^2 level indicating the existence of a classical inhibitory component of the $GABA_BR$ response involving VGCC inhibition and/or GIRK channel activation. This Ca^{2+}rise however is also potentiated by baclofen in ES cells. Hence, our results suggest that ES

cells express pharmacologically different $GABA_BR$ types that exert distinct functions through modulation of the $[Ca^{2+}]_i$ level.

GABAR as modulators of cell proliferation in ES cells

GABA has long been known as a modulator of cellular proliferation of neuronal progenitors (Barker et al., 1998; Haydar et al., 2000; Varju et al., 2001; Owens and Kriegstein, 2002b; Ben-Ari et al., 2007). Pioneer experiments showed that either GABA or $GABA_AR$ agonists inhibit cell proliferation in neocortical ventricular zone (VZ) cells (LoTurco et al., 1995). Similar results were obtained for adult neural stem cells (Liu et al., 2005) or cancer cells (Watanabe et al., 2006), while it stimulates proliferation of VZ cortical (Haydar et al., 2000) or cerebellar (Fiszman et al., 1999) progenitors, growth plate chondroctes (Tamayama et al., 2005), Schwann cell (Magnaghi et al., 2004), oligodendrocytes (Luyt et al., 2007) and cultured embryonic neural progenitor cells (Fukui et al., 2008).

Our results show, that co-activation of both GABA receptors in mES cells has little effect on the proliferation due to the opposite action of the two receptor types. Notably, the persistent activation of $GABA_AR$ has a differentiate effect based on the alkaline phosphatase staining experiments and induces expression of neuronal-specific markers. Thus, the action of GABA and different GABA ligands on proliferation and cell cycle are modulatory and not regulatory. Therefore, our results support the data obtained from GABAR knock-out experiments, which do not exert an overt phenotype in the preimplantation embryo (Burt, 2003)

1.4. GABA SIGNALLING DURING IN VITRO NEURONAL DIFFERENTIATION OF MOUSE ES CELL RECAPITULATION OF EMBRYONIC DEVELOPMENT

We found that *in vitro* model of neuronal differentiation of mouse ES cells (4-/4+) recapitulated many aspects of the normal development program that directs *in vivo* GABAergic differentiation. Our RT-PCR results revealed that all molecular components necessary for the assembly of functional GABA signalling follow a specific pattern of temporal regulation during different stages of neuronal differentiation. The embryonic and adult GAD forms were sequentially induced in the order GAD25, GAD44, GAD67 and GAD65 following the overlapping and transient expression of the upstream regulators, Dlx2 and Dlx5, respectively.

We detected 11 $GABA_A$ receptor subunits in our cultures: the ones whose genes are located on chromosome 4 (α2/4, β1 and γ1) and 15 (α5, β3 and γ3) were induced and upregulated during early stages (dai2, dai4) of neuronal differentiation and those located on chromosome 5 (α1, β2 and γ2L) were induced at a later differentiation period which is in agreement with data obtained in CNS. $GABA_B$ receptor subunits R1 and R2 were highly expressed in the first days after RA induction. Finally, the expression of both membrane (mostly GAT1) and vesicular GABA transporters (VGAT) appeared at early stages and increased in parallel with neuronal maturation, thus both vesicular and GAT-mediated GABA release may occur in the absence of synaptic specializations. As revealed by GABA immunocytochemistry, the earliest stages of differentiation were characterized by initial strong expression of GABA over all cells of the embryoid bodies (EBs), followed by gradual appearance first in bipolar cells, followed by a massive outmigration of GABAergic progenitors and sprouting of neuritis concomitant with the disappearance of staining from the EB remnant. These highly reproducible stages match the expression of a selective

repertoir of genes and may indicate the existence of a self-autonomous GABAergic differentiation programme.

2. DYNAMIC REGULATION OF MOLECULAR COMPONENTS AND FUNCTIONALITY OF THE GABA SIGNALING DURING MOUSE LENS DEVELOPMENT

The vertebrate lens develops from a series of interactions between the surface ectoderm, the optic vesicle, and the surrounding mesoderm, involving successive steps of bias, competence, specification, and differentiation (McAvoy, 1999; Hirch and Grainger, 2000). In addition to that, lens is attractive as a model system due to its regional compartmentalization. Proliferation occurs in the anterior lens epithelium, migration in the equatorial zone, and differentiation proceeds with cell elongation at the anterior/posterior poles.

Due to the above reasons, lens is a frequently used model to study to role of different signaling molecules in basic developmental processes.

Expression of different GAD forms recapitulates different phases of lens development

We found expression of all four known GAD forms during lens development : GAD65 and the three highly related proteins encoded by the GAD67 gene (mostly embryonic - GAD25, GAD44 and “adult” GAD67). During earlier embryonic stages (E14.5-E16.5) predominantly GAD65 and the embryonic forms GAD25 and GAD44 were expressed, adult GAD67 was only detected at a low level by RT-PCR, but not by Western blot, indicating that it may be present at extremely low levels. Our

immunohistochemistry results also showed that during E9.5-E16.5, the GAD65 and GAD67-related (mostly attributable to EGAD) immunolabeling was essentially overlapping, localized in both lens epithelium and lens fiber cells.

The highly coordinated expression of GAD65 and EGADs implies that these two forms have a synergistic role in the primary fiber differentiation (involving the processes of cell migration, elongation, and withdrawal from the cell cycle).

In accordance with their expression pattern, GAD44 along with GAD65 may provide GABA for the early stages of epithelial cell proliferation and primary fiber cell differentiation (***Fig.40***). 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 anteriorly and the lens capsule at the posterior lens wall. Similarly, the extremely high levels of "early" GADs at the anterior and posterior lens fiber tips and in the central lens epithelium during E13.5-E16.5 suggests that GABA synthesized by GAD44/GAD65 is involved in cell-cell adhesion mechanisms between fiber tips of opposite sides during formation of lens sutures (Fig.4, Kwakowski et al., 2007; Beebe et al., 2001). The highest level of expression of both EGADs and GAD65 was observed in the primary lens fibers at stages 16.5-E17.5 characterized by extensive fiber elongation and beginning of nuclear disintegration, indicating a role in this process as well. GAD65/EGAD sagregation of function may be linked to different mechanisms of GABA release, VGAT or GAT, respectively (***Fig.40***; Szabo et al., 1994).

The function of GAD25, the enzymatically inactive truncated form of glutamate decarboxylase, is more intriguing. Similar to the nervous system, GAD25 is

transiently expressed during early development, but it is also found in adult brain (Szabo et al., 1994, 2000).

From E17.5 on, GAD65, EGAD and GAD67 expression patterns change in accordance with the induction of primary fiber terminal differentiation and more enhanced secondary fiber generation, which continues into adult (***Fig.40***). GAD65 and EGADs gradually disappear from the lens core, concomitant with up-regulation of GAD67 at the equatorial region containing elongating secondary fibers. Therefore, it can be speculated that GAD67 is involved mostly in the differentiation of secondary fibers after birth.

Thus, there is a clear spatio-temporal segregation of "early" (GAD65 and EGAD) and "late" (GAD67) GAD forms. The predominant expression of GAD67 in the elongating nucleated fibers of the postnatal lens, where the lens maturation occurs much faster, in a narrow, 1-2 cell-wide region may be explained by the need for a quick recruitment of active GAD, which is provided by GAD67 existing in a PLP-bound holo-GAD, but not by GAD65, which is found mostly as an inactive apo-GAD (Martin et al., 2000; Varju et al., 2001) that is regulated by the PLP/ATP level in the cells.

Finally, the previously reported expression of GAD67 and embryonic forms in the developing rat lens (Li et al., 1995) shows remarkable similarities to that in the mouse with some notable differences: in E17.5 rat lens (corresponding roughly to E16.5 in the mouse), when the highest level of GAD is reached, mostly GAD67, but not EGAD is synthesized, furthermore, GAD67 was not detected before E15 and shortly after birth. Among these, the absence of GAD67 staining 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 immunoreactivity and the

presence of GABA in the postnatal rat lens (Li et al., 1995 and references therein). GABA has been detected at variable levels also in the lenses of other vertebrates (discussed in Li et al., 1995).

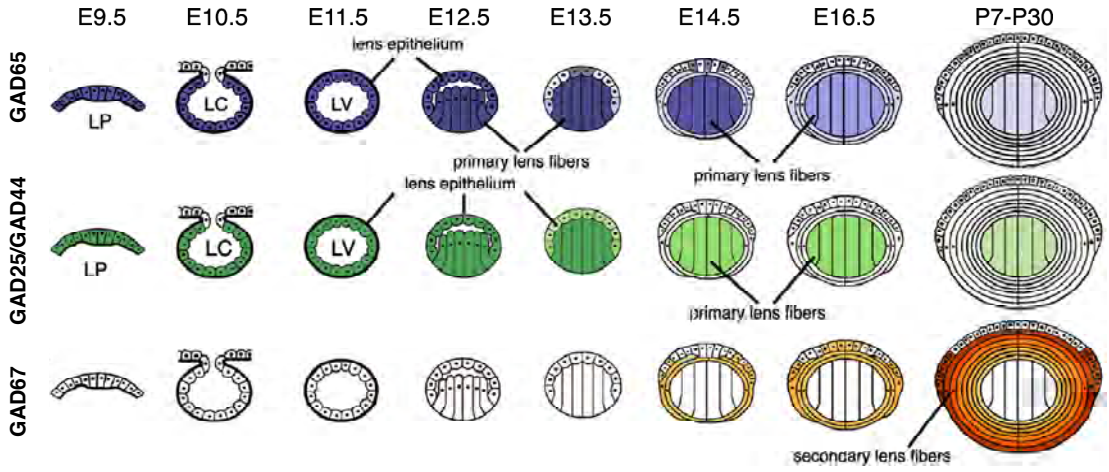


Fig. 40. Schematic presentation of GAD65 and GAD67 expression during lens development. Starting from the lens placode stage to E16.5, GAD65 (**Blue**) and GAD67-related (GAD25 and/or GAD44**; Green)** expression entirely overlaps. GAD65 expression overlaps with EGAD in lens epithelium and the primary fiber cells, where it is down- regulated after P14. At P30 and thereafter, only GAD67 (**Orange**) is expressed in the elongating secondary fiber cells and some cells of the epithelium, but not in lens nucleus.

2.2. Transcriptional regulation of GAD in the lens - involvement of Dlx2 and Dlx5

The earliest GAD (GAD65, EGAD) and GABA expression was observed in lens placode where multiple transcription factors like Pax6, Six3, Eya, Dach1, Mafs, Sox2 and 3 and Prox1 acting as key regulators in lens induction and fiber cell differentiation are present (see Introduction chapter, Fig. Kawauchi et al., 1999; Kim et al., 1999; Ogino and Yasuda, 2000; Ring et al., 2000; Purcell et al., 2005). Among these, Pax6 and Sox have binding sites on conserved regions of GAD65 and GAD67 promoters and are potential cis-regulators of GAD. Pax6, Six3 and Dach1 expression patterns (Purcell et al., 2005) largely overlap with GAD at early developmental stages not only in lens, but also in retina and ciliary marginal zone. Whether any of these

transcriptional factors directly regulate the transcription of GAD genes in the lens is a matter of speculation at present.

The Distal-less (Dlx) homeobox containing transcription factors also share overlapping domains of expression with GADs in the midgestation embryo (Katarova et al., 2000; Maddox and Condie, 2001), although it is not clear at present whether they can bind to and directly regulate GAD transcription. In the developing brain, GAD65 and GAD67 expression domains grossly overlap with that of the Dlx1-2 and Dlx5-6 implicated as key regulators of differentiation and migration of a subset of GABAergic inhibitory neurons (Anderson et al., 1997a,b; 1999; 2001; Cobos et al., 2005; Marin and Rubenstein, 2001; Katarova et al., 2000); Panganiban and Rubenstein, 2002; Pleasure et all., 2000). Dlx2 and Dlx5 may also regulate GAD in neurons outside the brain, like the migratory LHRH neurons, or in non-neuronal structures such as teeth, vibrissae, palate, where they show overlapping expression patterns (Porteus et al., 1994; Thomas et al., 1997; Katarova et al., 2000; Maddox and Condie, 2001; Depew et al., 2005; Givens et al., 2005; Tamayama et al., 2005; Levi et al., 2006).

The presence and possible function of Dlx2 and Dlx5 during lens development has not been described previously, although detailed expression studies in the mouse embryo have been published (reviewed in Panganiban and Rubenstein, 2002; Zerucha and Ekker, 2000). We find by semi-quantitive RT-PCR that the two genes are clearly expressed in an overlapping and sequential manner. Dlx2, which prevails at early stages, shows a temporal profile indistinguishable from that of EGAD and very similar to GAD65 may be needed for induction and/or maintenance of the early GAD forms (EGAD and GAD65). Dlx5 expression correlates only partially with that of GAD67: its initial up-regulation precedes that of GAD67 (E17.5 vs P0) and is not expressed beyond P14 in contrast to GAD67, which shows elevated expression until

P30. These findings strongly suggest that Dlx5 may be needed for induction, but not for maintenance of expression of GAD67. Further experiments will be needed to verify this hypothesis.

The sequential and overlapping expression of Dlx2 and Dlx5 in the lens is reminiscent to that in the brain (Liu et al., 1997; Eisenstat et al., 1999; Panganiban and Rubenstein, 2002) and may be related to the sequential induction of EGAD and GAD67 in the differentiating GABAergic progenitors (Katarova et al., 2000; Result Chapter 2.1). No lens phenotype has been described for Dlx1/2, Dlx5/6 double knock-out mutants, which exhibit embryonic or neonatal lethality (Zerucha and Ekker, 2000; Panganiban and Rubenstein, 2002; deMelo et al., 2005). This would suggest that Dlx genes have a subtle effect on lens morphogenesis during embryonic development, which can be better clarified in conditional knock-out experiments.

2.3. Different molecular components of the GABA signaling pathway exhibit similar expression profiles during lens development- evidence for co-regulation

We presented for the first evidence on the presence and dynamic regulation of all component of GABA signaling machinery involved in GABA synthesis (GAD), release (VGAT, GAT), binding ($GABA_A$ and $GABA_B$ receptors) and uptake (GAT) in the developing mouse lens.

We showed that selected $GABA_AR$ subunits ($\alpha_{1,2,3\ 4}$; $\beta_{1,\ 2,\ 3}$; $\gamma_{1,\ 2,\ 3}$ and δ), both $GABA_BR$ subunits, the vesicular and membrane GABA transporters are expressed in a strict spatio-temporal manner correlating with different stages 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 $\alpha_{,3}/\beta_3/\gamma_{1,2,3}/\delta$ 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 a_2/a_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 indicate that they are oppositely regulated at the transcriptional level throughout lens development. A coordinated transcriptional regulation has recently been demonstrated for the $\tilde{\beta_2}$-$\tilde{\alpha_6}$-$\tilde{\alpha_1}$-γ_2 subunit gene locus as well (Uusi-Oukari et al., 2000). The lens affords an ideal model to study such phenomena.

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 expression correlation data strongly suggest that in the embryonic lens GABA synthesized by GAD65 is released predominantly by VGAT and binds to $GABA_AR$ $a_{2,3}$ subunit-containing receptors, while that produced by GAD67 is released predominantly by the membrane GAT2 and binds to a_1-containing $GABA_AR$. GABA produced 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 high level of expression of GAT1-4 showing overlapping temporal profiles and especially the robust expression of GAT4 suggests that GAT-mediated GABA uptake/release coupled with Na^+/Cl^- transport performs important function(s) 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 (Schusboe et al., 2004). The developmentally earlier GAT3 is sorted to the apical membranes of polarized (Muth et al., 1998), and lens epithelia and apical/lateral membranes of differentiating lens fibers (Fig.3 and Fig.4, Kwakowski et al., 2008). 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 (Fig.3,

Kwakowski et al., 2007) 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 expression pattern of GAD, GABA, VGAT and GABA receptors highly resembles that of some typical neuronal synaptic vesicular transport-related proteins like synapsin, synaptotagmin, synaptophysin (Frederikse 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. 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.

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

In the adult brain, GABA, binding to GABA receptors exerts an inhibitory action, inducing membrane hyperpolarization and inhibition of Ca^{2+} currents. In immature brain activation of the ionotropic $GABA_AR$ induces membrane depolarization and Ca^{2+} influx through voltage-gated Ca^{2+} channels, while the metabotropic $GABA_BR$ is inhibitory, evoking membrane hyperpolarization and blockade (inhibition) of $[Ca^{2+}]_i$.

GABA receptors are also expressed on non-excitable cells, where GABA may acts as a trophic factor during the development similar to the CNS (Barker et al., 1998; Owens and Kriegstein, 2002a,2002b; Varju et al., 2001; Represa and Ben-Ari, 2005; Watanabe et al., 2002) but also non-neuronal tissues (pancreas, testis etc; Watanabe et al., 2002; Geigerseder et al., 2003; Franklin and Wollheim, 2004). In these structures GABA induces $[Ca^{2+}]_i$ rise either from internal stores or through voltage gated Ca^{2+} channels.

Using laser scanning confocal microscopy in freshly isolated P0 lenses loaded with Fluo-3/AM we detected simultaneous rise in $[Ca^{2+}]_i$ in epithelial cells and elongating fibers of the equatorial region upon bath application of GABA, indicating the existence of active GABA receptor response in this region.

To characterize the GABA receptor–induced calcium transients we have expanded this approach to an *in vitro* model system: detection of $[Ca^{2+}]_i$. changes in primary LEC cultures using selective GABA receptor agonists/antagonists and GABA. To date, lens cell lines and primary cultures derived from several species have been successfully used to study aspects of fiber cell differentiation and Ca^{2+}signaling pathways (Duncan et al., 1993; Churchill and Louis, 1998). The method, developed by us, was a novel approach by which we could obtain of primary LEC at different differentiation stages (epithelial cells, nucleated and anucleated fibers) found in native lenses and in addition they were far more permeable for Ca^{2+} imaging dyes than the native lens. Furthermore, most of the lens epithelial cells and lentoids, isolated from newborn mice and grown for two weeks in culture, expressed molecular components of GABA signaling: GAD, GABA transporters and GABA receptors found in the lens.

Surprisingly, both muscimol ($GABA_AR$ agonist) and baclofen ($GABA_BR$ agonist) evoked Ca^{2+} transients, predominantly in lentoids and sheets of epithelial cells, but

also in single cells, that could be blocked by the selective antagonists bicuculline and CGP55845, respectively. Whereas the $GABA_AR$-mediated Ca^{2+} rise in LEC is entirely consistent with the channel properties in the embryonic CNS (Ben-Ari et al., 2007) and/or non-neuronal cells (Watanabe et al., 2002; Franklin and Wollheim, 2004), the $GABA_BR$- evoked responses were unexpected, as it is widely considered to be inhibitory in both embryo and adult (Owens and Kriegstein, 2002; 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. 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.

Moreover, we have been able to record two additional specific features of the LEC responses - a synchronous reaction of cell groups, more pronounced at $GABA_BR$ activation, which is often followed by wave-propagation of the signal and persistent synchronous oscillations and delayed Ca^{2+} transients, observed often upon GABA or baclofen, but never by muscimol applications. This wave-propagation of the signal strongly suggests the involvement of gap junctions known to mediate Ca^{2+} waves in primary LEC (Churchill et al., 1996,1998). Interestingly, persistent Ca^{2+} elevation (but not cell-to-cell Ca^{2+} waves) causes gap junction uncoupling and formation of

hemichannels, implying that GABA, acting on GABAR may regulate gap junction formation through $[Ca^{2+}]_i$. and thus ultimately influence the gap-junction-mediated lens transport.

The GABA signaling described here is a novel neurotransmitter signaling system described 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 (Duncan and Collison, 2003 ; Oppitz et al., 2003). Based on previous (Oppitz et al., 2003) and present 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 neuronal progenitors that may provide for compensatory mechanisms during early development (Owens and Kriegstein, 2002). Another important feature of LEC is the GABAR (predominantly $GABA_BR$)- induced oscillation as it may have a long-term effect on the cell cycle and differentiation similar to the GABAR-mediated oscillations described for the gap junction-coupled embryonic neuroepithelial cells (LoTurco et al., 1995; Owens and Kriegstein, 1998; 2002).

2.4. Lens as a model to study the non-synaptic GABA signaling during early steps of differentiation

Our results show that all molecular components needed to form a functional GABA signaling system are expressed from early stages of lens development in a strict spatio-temporal manner, which parallels different phases of lens cell differentiation

suggesting that GABA may play diverse stage-specific function in this process. Structurally, GABA synthesis (GAD), release (VGAT, GAT) and GABAR binding 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.41***).

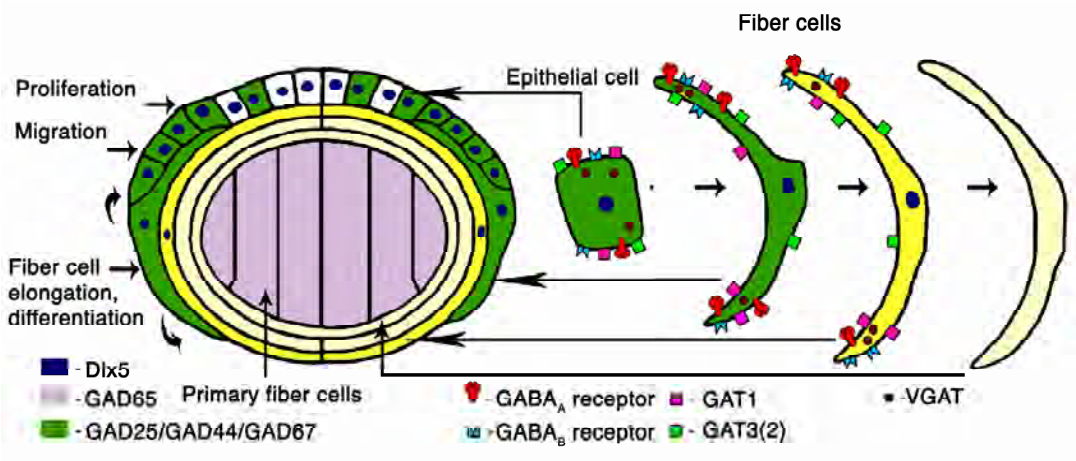


Fig.41. Expression patterns of different GABA signaling components in the neonatal mouse lens: a schematic presentation. GAD65 is localized almost exclusively in the primary lens fibers comprising the lens nucleus, while GAD67 is mostly found in the elongating secondary fibers and lens epithelium cells. 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.

Our results suggest that GABA, predominantly located in the germinative and migratory zones of the lens epithelium and elongating primary or secondary fibers, 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 (see Introduction). 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.

The fiber cell differentiation in the lens is thought to proceed through an unusual, attenuated apoptosis (Ishizaki et al., 1998; Wride, 2000; Bassnett et al., 2002; Zandy et al., 2005; Weber and Menko, 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.

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. Therefore, GABA pools synthesized by different GAD enzyme and released by distinct mechanisms may influence differentially the intercellular communications through adherens and gap junctions and/or reorganization of the membrane skeleton, both Ca^{2+}-dependent processes (discussed in the previous chapter). Selective sorting of the GABA transporters to the apical/basal surfaces, an equivalent of the axonal/dendritic neuronal compartments may have an active role in the osmo- and cell-volume regulation (Schousboe et al., 2004). Similar role can be proposed for the lens based on the high level of GAT/GABA expression at stages of most intensive fiber elongation.

In conclusion, the existence of GABA signaling machinery in the developing lens would suggest that it may be a target to a variety of drug treatments acting

predominantly on GABAR like benzodiazepines, hormones and neurosteroids and also in certain diseases affecting the GABA signaling, like IDDM. Furthermore, the lens is a dynamic organ that continues to grow throughout development and calcium signaling plays a key role in it. Several lines of evidence implicate the loss of calcium homeostasis in the lens as key factor in cataract formation (reviewed in Duncan and Wormstone, 1999). The loss of transparency during cataract development severely impairs the ability of the lens to focus on the image on the retina and is the leading cause of blindness worldwide. It would be important to study the effect of such treatments or pathological conditions on the lens transparency in order to develop preventive strategies.

VIII CONCLUSIONS

- Undifferentiated pluripotent embryonic stem (ES) cells express active GABA signalling system assembled from neuron-specific components- GAD, GABAR, GABA transporters and uptake.
- The temporal regulation of the two known GAD genes coding for the GABA-synthesizing enzymes GAD65 and GAD67 and the upstream transcriptional regulators Dlx2/5 follows a highly reproducible paradigm in different in vivo and in vitro differentiation systems.
- Based on our expression analysis, both vesicular and membrane transporter-mediated GABA release may occur in undifferentiated ES cells and during neuronal or lens differentiation, in the absence of inhibitory synapses.
- $GABA_A$ and $GABA_B$ receptors in ES cells and lens epithelial cells display unique composition and properties; both receptor types evoke intracellular $Ca2^+$ rise by distinct mechanisms involving both the extracellular Ca (VGCC) and intracellular Ca stores (G-proteins).
- Despite the synergistic effect on the intracellular Ca^{2+} exerted by $GABA_A$ and $GABA_B$ receptors, they exert opposite effects on cellular proliferation and/or differentiation of ES cells. This effect is in sharp contrast to the receptors' properties in immature neurons, but may be similar to these in neural stem cells.
- GABA may serve as a paracrine –autocrine signaling molecule at very first stages of embryonic development affecting the cell proliferation, cell-cell communications and/or differentiation by mechanism(s) similar to that in the embryonic nervous system.
- Due to the spatio-temporal segregation of GABA signalling components along the two GAD proteins, the lens has proven a unique model system affording

the possibility to study the specific roles of GAD65 and GAD67 and components downstream of each gene, especially in view of their correlated regulation during development and in the adult.

ORIGINAL PAPERS USED FOR THE THESIS:

1. Kwakowsky, A*., **M. Schwirtlich***, Q. Zhang, D. D. Eisenstat, F. Erdelyi, M. Baranyi, Z. D. Katarova and G. Szabo (2007). "GAD isoforms exhibit distinct spatiotemporal expression patterns in the developing mouse lens: correlation with Dlx2 and Dlx5." Dev Dyn **236**(12): 3532-44.

2. Kwakowsky, A*.,**M. Schwirtlich*,** F. Kooy, I. Abraham, Z. Mate, Z. Katarova and G. Szabo (2008). "GABA neurotransmitter signaling in the developing mouse lens: dynamic regulation of components and functionality." Dev Dyn **237**(12): 3830-41

3. **Schwirtlich M,** Zs Emri, K Antal K, Z Máté, Z Katarova and G Szabó Activation of $GABA_A$ and $GABA_B$ receptors of distinct properties adversely affects proliferation of mouse embryonic stem (ES) cells through modulation of intracellular Ca^{2+}signalling. *Manuscript under revision.*

* Authors contributed equally

IX SUMMARY

γ - aminobutyric acid (GABA) is the main inhibitory neurotransmitter in the CNS and as such regulates neuronal excitability. During development GABA acts as a trophic factor modulating processes such as proliferation, differentiation and migration in both neuronal and non-neuronal tissues. The aim of the present study was to characterize GABA signaling components and function during development in two different model systems: mouse embryonic stem (ES) cells and the ocular lens. Our results can be summarized as:

1. Undifferentiated pluripotent embryonic stem (ES) cells and differentiating lens cells express neuron-specific components necessary for an active GABA signaling system including GAD, GABAR and GABA transporters.
2. The temporal regulation of the two known GAD genes coding for the GABA-synthesizing enzymes GAD65 and GAD67 and the upstream transcriptional regulators Dlx2/5 follows a highly reproducible pattern in the studied in vivo and in vitro differentiation systems.
3. Based on our expression analysis, both vesicular and membrane transporter-mediated GABA release may occur in undifferentiated ES cells and during neuronal or lens differentiation in the absence of inhibitory synapses.
4. $GABA_A$ and $GABA_B$ receptors in ES cells and lens epithelial cells display unique composition and properties; both receptor types evoke intracellular Ca^{2+} rise by distinct mechanisms involving both the extracellular Ca^{2+} and intracellular Ca^{2+} stores.
5. Despite the synergistic effect of $GABA_A$ and $GABA_B$ receptors on the intracellular Ca^{2+}-concentration, they exert opposite effects on cellular proliferation and/or differentiation of ES cells. This effect is in sharp contrast to the receptors' properties in immature neurons, but may be similar to these in neural stem cells.

Our findings supports the view that GABA may serve as a paracrine–autocrine signaling molecule at early stages of embryonic development affecting cell proliferation, cell-cell communications and/or differentiation by mechanism(s) similar to that in the embryonic nervous system.

Using these two model systems will allow us to uncover the general mechanism of GABA action in basic developmental processes, which could also apply to the nervous system.

ÖSSZEFOGLALÁS

A gamma-aminovajsav (GABA) a központi idegrendszer legfontosabb gátló neurotranszmittereként kulcsszerepet játszik az idegsejtek ingerelhetőségének szabályozásában.

A GABA-nak az idegrendszer és a nem-idegi szövetek fejlődése során pedig szerepe van a proliferáció, differenciáció és migráció szabályozásában is. A disszertáció alapját képező kísérleteink legfőbb célja az volt, hogy jellemezzük a GABA jelátvitel összetevőit és azok működését az egyedfejlődés során. Ehhez két modellrendszert, az egér differenciálatlan embrionális őssejteket és a fejlődő szemlencsét használtuk. Jelentősebb eredményeink az alábbiakban foglalhatók össze:

1. A GABA jelátviteli folyamat összes komponense: a GABA, GAD, $GABA_A$, $GABA_B$ receptorok valamint a GABA transzporterek jelen vannak az ES sejtekben és a fejlődő szemlencsében is, mintegy megteremtve a GABA szintézis, kibocsátás, érzékelés és újrafelvétel lehetőségét.
2. A GABA-szintetizáló enzimeket kódoló gének (GAD65 és GAD67) és azok szabályozásában szerepet játszó Dlx2/5 gének időbeli expressziós mintázata nagyon hasonló az általunk vizsgált *in vitro* és *in vivo* differenciációs rendszerekben.
3. Az expressziós vizsgálatunk eredményei szerint a vezikuláris és membrántranszport-mediált GABA-felszabadulás egyaránt előfordulhat a differenciálatlan ES sejtekben és a fejlődő szemlencsében is.
4. A differenciálatlan ES sejtekben és a lencse hámsejtekben a $GABA_A$ és $GABA_B$ receptorok sajátos alegység összetétellel és funkcionális sajátosságokkal rendelkeznek. Mindkét receptortípus aktiválódása sejtenbelüli kálcium-szint növekedést okoz, amelynek a mechanizmusa eltérő, és a Ca^{2+} mind az extracelluláris térből, mind pedig a belső raktárokból is származhat.
5. Annak ellenére, hogy a $GABA_A$ és $GABA_B$ receptorok hasonlóan befolyásolják az intracelluláris Ca^{2+}-koncentrációt, a sejtosztódásra és differenciációra gyakorolt hatásuk ellentétes. Ez különbözik az éretlen neuronokban kifejtett hatástól, de hasonló lehet az ideg őssejtekben zajló folyamatokhoz.

Eredményeink alátámasztják azt a feltételezést, hogy a GABA auto- illetve parakrin faktorként befolyásolhatja a sejtosztódást, differenciációt és a sejtek közötti kommunikációt a korai embrionális fejlődés során.

Modellrendszereink segítségével kideríthetjük a GABA hatás pontos mechanizmusát a fejlődési folyamatokban.

X ACKNOWLEDGEMENTS

It is a pleasure to thank the many people who made this thesis possible.
First, I would like to express my sincere gratitude to supervisor ***Dr. Gábor Szábo*** *for enormous patience and warm support. You gave me the chance to join your research group and to work in a stimulating atmosphere to very high standards. You were always there to listen and to give advices. You taught me, at least I hope, how to ask question and express my ideas.*
It is a difficult to overstate my gratitude to ***Dr Zoja Katarova*** *for being our "promoter", great inspiration, teacher and advisor for all these years. Zoja showed me different ways to approach a research problem and need to be persistent to accomplish the goal. Her ideas and tremendous support had a major influence on this thesis.*
Here, I also would like to thank ***Dr. Mirjana Stojiljkovic****, who was my supervisor during my undergraduate years, who introduced me to the field of neuroscience, and who latter continued to provide further encouragement.*
Many thanks to my friends and colleagues from the ***Laboratory of Molecular Biology and Genetics*** *for the great time I had in the group over the years:* ***Zoltán Máté*** *and* ***Csaba Vastagh****, for all "immediately necessary" help you provided,* ***Dr. Ildikó Szatmári****,* ***Dr. Zsolt Lele*** *and* ***Dr. Ferenc Erdélyi*** *for the interesting and fruitful discussions we had during "short" coffee breaks.*
Especially, thanks to my lab partner, ***Andrea Kwakowski*** *for sharing weekends, hot chocolates and many "miserable" life stories. We also fought a lot being both stubborn, but in the end, we accomplished a lot in describing the GABA signaling pathway in the mouse lens. My keen appreciation goes to* ***Bogáta Kovács, László Dezső. Katalin Döme, Emőke Lukácsi, and Andrea Jurász*** *for their valuable assistance. Without their help, the experimental work would not have been accomplished in time.*
Sincere thanks are extended to the entire stuff of the "Animal house", especially ***Dr. Balázs Bényei, Mária Szűcs*** *and* ***Rozália Szafner*** *without whose cooperation and help this work would not be possible.*
Many thanks to all my colleagues in the ***Institute of Experimental Medicine*** *for the friendly, intellectual and stimulating environment. It was an honour to be part of the "KOKI".*

***Anna Földes**, thank you for just being THE ANNA**. Aniko Ludany**, thank you for all Coca Cola or popcorn breaks, which should always happened "immediately" or "now".*

*I wish to express my cordial Appreciaiation to our collaborators: **Dr. Zsuzsa Emri**, **Dr. Elen Gócza**, **Dr. István Ábrahám**, **Dr. Mária Baranyi**, and **Dr. David Eisenstadt**.*

*I am also indebted to **Marc Eyre** for devoting his precious time and made valuable suggestions, which indeed helped improve of this thesis.*

*I would like to thank my **friends** for their support and enthusiasm, which was constant and overlay supporting in course of the work. Especially I want to thank my best friend **Saša**, who made "anything - anytime" possible and/or available.*

*Lastly, and most importantly, I wish to thank my parents, **Neda** and **Laszlo Schwirtlich** for unconditional support and encouragement to pursue my interests, even when the interest went beyond boundaries of language, field and geography. To them I dedicate this thesis.*

Thank you.

XI BIBLIOGRAPHY

1. **Agmo, A.,** *R. Paredes, and H. Fernandez,* Differential effects of GABA transaminase inhibitors on sexual behavior, locomotor activity, and motor execution in the male rat. *Pharmacol Biochem Behav, 1987.* ***28****(1): p. 47-52.*
2. **Aguado, C.,** *et al.,* Cell type-specific subunit composition of G protein-gated potassium channels in the cerebellum. *J Neurochem, 2008.* ***105****(2): p. 497-511.*
3. **Alcala, J.,** *N. Lieska, and H. Maisel,* Protein composition of bovine lens cortical fiber cell membranes. *Exp Eye Res, 1975.* ***21****(6): p. 581-95.*
4. **Anderson, S.A.,** *et al.,* Interneuron migration from basal forebrain to neocortex: dependence on Dlx genes. *Science, 1997.* ***278****(5337): p. 474-6.*
5. **Anderson, S.A.,** *et al.,* Mutations of the homeobox genes Dlx-1 and Dlx-2 disrupt the striatal subventricular zone and differentiation of late born striatal neurons. *Neuron, 1997.* ***19****(1): p. 27-37.*
6. **Armstrong, L.,** *et al.,* mTert expression correlates with telomerase activity during the differentiation of murine embryonic stem cells. *Mech Dev, 2000.* ***97****(1-2): p. 109-16.*
7. **Attwell, D.,** *B. Barbour, and M. Szatkowski,* Nonvesicular release of neurotransmitter. *Neuron, 1993.* ***11****(3): p. 401-7.*
8. **Azuma, H.,** *et al.,* Gamma-aminobutyric acid as a promoting factor of cancer metastasis; induction of matrix metalloproteinase production is potentially its underlying mechanism. *Cancer Res, 2003.* ***63****(23): p. 8090-6.*
9. **Bain, G.,** *et al.,* Embryonic stem cells express neuronal properties in vitro. *Dev Biol, 1995.* ***168****(2): p. 342-57.*
10. **Bain, G.,** *et al.,* Retinoic acid promotes neural and represses mesodermal gene expression in mouse embryonic stem cells in culture. *Biochem Biophys Res Commun, 1996.* ***223****(3): p. 691-4.*
11. **Balakrishnan, V.,** *et al.,* Expression and function of chloride transporters during development of inhibitory neurotransmission in the auditory brainstem. *J Neurosci, 2003.* ***23****(10): p. 4134-45.*
12. **Balasubramanian, S.,** *et al.,* Hetero-oligomerization between GABAA and GABAB receptors regulates GABAB receptor trafficking. *J Biol Chem, 2004.* ***279****(18): p. 18840-50.*
13. **Barbin, G.,** *et al.,* Involvement of GABAA receptors in the outgrowth of cultured hippocampal neurons. *Neurosci Lett, 1993.* ***152****(1-2): p. 150-4.*
14. **Barker, J.L.,** *et al.,* GABAergic cells and signals in CNS development. *Perspect Dev Neurobiol, 1998.* ***5****(2-3): p. 305-22.*
15. **Barnard, E.A.,** *et al.,* Multiplicity, structure, and function in GABAA receptors. *Ann N Y Acad Sci, 1993.* ***707****: p. 116-25.*

16. **Bassnett, S.,** Lens organelle degradation. *Exp Eye Res, 2002. **74**(1): p. 1-6.*
17. *Bassnett, S.,* On the mechanism of organelle degradation in the vertebrate lens. *Exp Eye Res, 2008.*
18. ***Battaglioli,** G., H. Liu, and D.L. Martin,* Kinetic differences between the isoforms of glutamate decarboxylase: implications for the regulation of GABA synthesis. *J Neurochem, 2003. **86**(4): p. 879-87.*
19. ***Baumann, S.W.,** R. Baur, and E. Sigel,* Subunit arrangement of gamma-aminobutyric acid type A receptors. *J Biol Chem, 2001. **276**(39): p. 36275-80.*
20. ***Beebe, D.C.,** et al.,* Changes in adhesion complexes define stages in the differentiation of lens fiber cells. *Invest Ophthalmol Vis Sci, 2001. **42**(3): p. 727-34.*
21. ***Behar, T.,** et al.,* Analysis of the anatomical distribution of GAD67 mRNA encoding truncated glutamic acid decarboxylase proteins in the embryonic rat brain. *Brain Res Dev Brain Res, 1994. 77(1): p. 77-87.*
22. ***Behar, T.N.,** et al.,* GABA receptor antagonists modulate postmitotic cell migration in slice cultures of embryonic rat cortex. *Cereb Cortex, 2000. **10**(9): p. 899-909.*
23. ***Behar, T.N.,** et al.,* Differential response of cortical plate and ventricular zone cells to GABA as a migration stimulus. *J Neurosci, 1998. **18**(16): p. 6378-87.*
24. ***Behar, T.N**., et al.,* GABA(B) receptors mediate motility signals for migrating embryonic cortical cells. *Cereb Cortex, 2001. **11**(8): p. 744-53.*
25. ***Ben-Ari, Y.,*** Excitatory actions of gaba during development: the nature of the nurture. *Nat Rev Neurosci, 2002. **3**(9): p. 728-39.*
26. ***Ben-Ari, Y.**, et al.,* GABA: a pioneer transmitter that excites immature neurons and generates primitive oscillations. *Physiol Rev, 2007. **87**(4): p. 1215-84.*
27. ***Berridge, M.J.**, M.D. Bootman, and H.L. Roderick,* Calcium signalling: dynamics, homeostasis and remodelling. *Nat Rev Mol Cell Biol, 2003. **4**(7): p. 517-29.*
28. ***Bettler, B.,** et al.,* Molecular structure and physiological functions of GABA(B) receptors. *Physiol Rev, 2004. **84**(3): p. 835-67.*
29. ***Bhat, S.P.,*** Crystallins, genes and cataract. *Prog Drug Res, 2003. **60**: p. 205-62.*
30. ***Bianchi, L.**, et al.,* Extracellular levels of amino acids and choline in human high grade gliomas: an intraoperative microdialysis study. *Neurochem Res, 2004. **29**(1): p. 325-34.*
31. ***Biju, M.P.,** et al.,* Enhanced GABA(B) receptor in neoplastic rat liver: induction of DNA synthesis by baclofen in hepatocyte cultures. *J Biochem Mol Biol Biophys, 2002. **6**(3): p. 209-14.*
32. ***Bloemendal, H.,** et al.,* Ageing and vision: structure, stability and function of lens crystallins. *Prog Biophys Mol Biol, 2004. **86**(3): p. 407-85.*
33. ***Bloemendal, M**., et al.,* A structural study of bovine lens alpha-crystallin and its subunits by absorption and linear dichroism spectroscopy. *Eur J Biochem, 1989. **184**(2): p. 427-32.*
34. ***Bloom, F.E.** and L.L. Iversen,* Localizing 3H-GABA in nerve terminals of rat cerebral cortex

by electron microscopic autoradiography. *Nature, 1971.* ***229****(5287): p. 628-30.*

35. ***Bollan, K.A.****, et al.,* The promiscuous role of the epsilon subunit in GABAA receptor biogenesis. *Mol Cell Neurosci, 2008.* ***37****(3): p. 610-21.*

36. ***Bond, R.W.,*** *R.J. Wyborski, and D.I. Gottlieb,* Developmentally regulated expression of an exon containing a stop codon in the gene for glutamic acid decarboxylase. *Proc Natl Acad Sci U S A, 1990.* ***87****(22): p. 8771-5.*

37. ***Borden, L.A.,*** GABA transporter heterogeneity: pharmacology and cellular localization. *Neurochem Int, 1996.* ***29****(4): p. 335-56.*

38. ***Bormann, J****.,* The 'ABC' of GABA receptors. *Trends Pharmacol Sci, 2000.* ***21****(1): p. 16-9.*

39. ***Bouche, N.*** *and H. Fromm,* GABA in plants: just a metabolite? *Trends Plant Sci, 2004.* ***9****(3): p. 110-5.*

40. ***Bouche, N., B****. Lacombe, and H. Fromm,* GABA signaling: a conserved and ubiquitous mechanism. *Trends Cell Biol, 2003.* ***13****(12): p. 607-10.*

41. ***Bowery, N.G.,*** GABAB receptor: a site of therapeutic benefit. *Curr Opin Pharmacol, 2006.* ***6****(1): p. 37-43.*

42. ***Bowery, N.G.,*** *et al.,* International Union of Pharmacology. XXXIII. Mammalian gamma-aminobutyric acid(B) receptors: structure and function. *Pharmacol Rev, 2002.* ***54****(2): p. 247-64.*

43. ***Bowery, N.G****. and D.A. Brown,* The cloning of GABA(B) receptors. *Nature, 1997.* ***386****(6622): p. 223-4.*

44. ***Braun, M.,*** *et al.,* Regulated exocytosis of GABA-containing synaptic-like microvesicles in pancreatic beta-cells. *J Gen Physiol, 2004.* ***123****(3): p. 191-204.*

45. ***Bulfone, A.,*** *et al.,* Spatially restricted expression of Dlx-1, Dlx-2 (Tes-1), Gbx-2, and Wnt-3 in the embryonic day 12.5 mouse forebrain defines potential transverse and longitudinal segmental boundaries. *J Neurosci, 1993.* ***13****(7): p. 3155-72.*

46. ***Burt, D.R****.,* Reducing GABA receptors. *Life Sci, 2003.* ***73****(14): p. 1741-58.*

47. ***Buznikov, G.A.,*** *Y.B. Shmukler, and J.M. Lauder,* From oocyte to neuron: do neurotransmitters function in the same way throughout development? *Cell Mol Neurobiol, 1996.* ***16****(5): p. 537-59.*

48. ***Calver, A.R.,*** *et al.,* The expression of GABA(B1) and GABA(B2) receptor subunits in the cNS differs from that in peripheral tissues. *Neuroscience, 2000.* ***100****(1): p. 155-70.*

49. ***Carter, T.J****. and M. Mynlieff,* Gamma-aminobutyric acid type B receptors facilitate L-type and attenuate N-type Ca(2+) currents in isolated hippocampal neurons. *J Neurosci Res, 2004.* ***76****(3): p. 323-33.*

50. ***Chambliss, K.L****., et al.,* Two exon-skipping mutations as the molecular basis of succinic semialdehyde dehydrogenase deficiency (4-hydroxybutyric aciduria). *Am J Hum Genet, 1998.* ***63****(2): p. 399-408.*

51. ***Chaudhry, F.A.,*** *et al.,* The vesicular GABA transporter, VGAT, localizes to synaptic

vesicles in sets of glycinergic as well as GABAergic neurons. *J Neurosci, 1998.* ***18****(23): p. 9733-50.*

52. ***Chen, Y.,*** *et al.*, Study on olfactory function in GABAC receptor/channel rho1 subunit knockout mice. *Neurosci Lett, 2007.* ***427****(1): p. 10-5.*

53. ***Choi, J.,*** *S.Y. Park, and C.K. Joo,* Hepatocyte growth factor induces proliferation of lens epithelial cells through activation of ERK1/2 and JNK/SAPK. *Invest Ophthalmol Vis Sci, 2004.* ***45****(8): p. 2696-704.*

54. ***Chomczynski, P****. and N. Sacchi,* Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal Biochem, 1987.* ***162****(1): p. 156-9.*

55. ***Churchill, G.C.,*** *M.M. Atkinson, and C.F. Louis,* Mechanical stimulation initiates cell-to-cell calcium signaling in ovine lens epithelial cells. *J Cell Sci, 1996.* ***109 (Pt 2)****: p. 355-65.*

56. ***Churchill, G.C****. and C.F. Louis,* Roles of Ca2+, inositol trisphosphate and cyclic ADP-ribose in mediating intercellular Ca2+ signaling in sheep lens cells. *J Cell Sci, 1998.* ***111 (Pt 9)****: p. 1217-25.*

57. ***Cohen, S.M.,*** *et al.*, Distal-less encodes a homoeodomain protein required for limb development in Drosophila. *Nature, 1989.* ***338****(6214): p. 432-4.*

58. ***Cohen, S.M****. and G. Jurgens,* Proximal-distal pattern formation in Drosophila: cell autonomous requirement for Distal-less gene activity in limb development. *Embo J, 1989.* ***8****(7): p. 2045-2055.*

59. ***Collinson, N.,*** *et al.*, Enhanced learning and memory and altered GABAergic synaptic transmission in mice lacking the alpha 5 subunit of the GABAA receptor. *J Neurosci, 2002.* ***22****(13): p. 5572-80.*

60. ***Conti, F.,*** *A. Minelli, and M. Melone,* GABA transporters in the mammalian cerebral cortex: localization, development and pathological implications. *Brain Res Brain Res Rev, 2004.* ***45****(3): p. 196-212.*

61. ***Cossart, R.,*** *et al.*, Interneurons targeting similar layers receive synaptic inputs with similar kinetics. *Hippocampus, 2006.* ***16****(4): p. 408-20.*

62. ***Costa, E.,*** From GABAA receptor diversity emerges a unified vision of GABAergic inhibition. *Annu Rev Pharmacol Toxicol, 1998.* ***38****: p. 321-50.*

63. ***Coull, J.A****., et al.,* Trans-synaptic shift in anion gradient in spinal lamina I neurons as a mechanism of neuropathic pain. *Nature, 2003.* ***424****(6951): p. 938-42.*

64. ***Couve, A.,*** *et al.*, Unravelling the unusual signalling properties of the GABA(B) receptor. *Biochem Pharmacol, 2004.* ***68****(8): p. 1527-36.*

65. ***Couve, A.,*** *et al.*, Intracellular retention of recombinant GABAB receptors. *J Biol Chem, 1998.* ***273****(41): p. 26361-7.*

66. ***Couve, A.,*** *S.J. Moss, and M.N. Pangalos,* GABAB receptors: a new paradigm in G protein signaling. *Mol Cell Neurosci, 2000.* ***16****(4): p. 296-312.*

67. ***de Melo, J.,*** *et al.*, Dlx1 and Dlx2 function is necessary for terminal differentiation and

survival of late-born retinal ganglion cells in the developing mouse retina. *Development, 2005.* ***132****(2): p. 311-22.*

68. ***DeLorey, T.M****. and R.W. Olsen,* GABA and epileptogenesis: comparing gabrb3 gene-deficient mice with Angelman syndrome in man. *Epilepsy Res, 1999.* ***36****(2-3): p. 123-32.*

69. ***Depew, M.J.,*** *et al.,* Reassessing the Dlx code: the genetic regulation of branchial arch skeletal pattern and development. *J Anat, 2005.* ***207****(5): p. 501-61.*

70. ***Ding, R., N.*** *Tsunekawa, and K. Obata,* Cleft palate by picrotoxin or 3-MP and palatal shelf elevation in GABA-deficient mice. *Neurotoxicol Teratol, 2004.* ***26****(4): p. 587-92.*

71. ***Docherty, M.,*** *et al.,* Evidence for specific immunolysis of nerve terminals using antisera against choline acetyltransferase, glutamate decarboxylase and tyrosine hydroxylase. *Brain Res, 1985.* ***339****(1): p. 105-13.*

72. ***Drescher, D.G.,*** *et al.,* Analysis of gamma-aminobutyric acidA receptor subunits in the mouse cochlea by means of the polymerase chain reaction. *J Neurochem, 1993.* ***61****(3): p. 1167-70.*

73. ***Duncan, G.*** *and D.J. Collison,* Role of the non-neuronal cholinergic system in the eye: a review. *Life Sci, 2003.* ***72****(18-19): p. 2013-9.*

74. ***Duncan, G.****, et al.,* Calcium regulation in tissue-cultured human and bovine lens epithelial cells. *Invest Ophthalmol Vis Sci, 1993.* ***34****(10): p. 2835-42.*

75. ***Duncan, G****. and I.M. Wormstone,* Calcium cell signalling and cataract: role of the endoplasmic reticulum. *Eye, 1999.* ***13 (Pt 3b)****: p. 480-3.*

76. ***Eisenstat, D.D.,*** *et al.,* DLX-1, DLX-2, and DLX-5 expression define distinct stages of basal forebrain differentiation. *J Comp Neurol, 1999.* ***414****(2): p. 217-37.*

77. ***Erdo, S.L****. and F. Amenta,* Characterization and localization of high-affinity GABA uptake in slices of the rabbit oviduct. *Eur J Pharmacol, 1986.* ***130****(3): p. 287-94.*

78. ***Erdo, S.L.*** *and F. Amenta,* Specific, vascular localization of GABA-transaminase in the guinea pig lung. *Neurosci Lett, 1986.* ***68****(2): p. 202-6.*

79. ***Erdo, S.L.,*** *F. Joo, and J.R. Wolff,* Immunohistochemical localization of glutamate decarboxylase in the rat oviduct and ovary: further evidence for non-neural GABA systems. *Cell Tissue Res, 1989.* ***255****(2): p. 431-4.*

80. ***Erdo, S.L.*** *and J.R. Wolff,* gamma-Aminobutyric acid outside the mammalian brain. *J Neurochem, 1990.* ***54****(2): p. 363-72.*

81. ***Erlander, M.G.,*** *et al.,* Two genes encode distinct glutamate decarboxylases. *Neuron, 1991.* ***7****(1): p. 91-100.*

82. ***Erlitzki, R.,*** *et al.,* Identification of gamma-aminobutyric acid receptor subunit types in human and rat liver. *Am J Physiol Gastrointest Liver Physiol, 2000.* ***279****(4): p. G733-9.*

83. ***Esclapez, M.,*** *et al.,* Comparative localization of two forms of glutamic acid decarboxylase and their mRNAs in rat brain supports the concept of functional differences between the forms. *J Neurosci, 1994.* ***14****(3 Pt 2): p. 1834-55.*

84. **Etheredge, J.A.,** *et al.,* Functional compensation by other voltage-gated Ca2+ channels in mouse basal forebrain neurons with Ca(V)2.1 mutations. *Brain Res, 2007.* ***1140****: p. 105-19.*

85. **Evans, M.J.** *and M.H. Kaufman,* Establishment in culture of pluripotential cells from mouse embryos. *Nature, 1981.* ***292****(5819): p. 154-6.*

86. **Farrant, M.** *and K. Kaila,* The cellular, molecular and ionic basis of GABA(A) receptor signalling. *Prog Brain Res, 2007.* ***160****: p. 59-87.*

87. **Fiszman, M.L.,** *L.N. Borodinsky, and J.H. Neale,* GABA induces proliferation of immature cerebellar granule cells grown in vitro. *Brain Res Dev Brain Res, 1999.* ***115****(1): p. 1-8.*

88. **Fon, E.A.** *and R.H. Edwards,* Molecular mechanisms of neurotransmitter release. *Muscle Nerve, 2001.* ***24****(5): p. 581-601.*

89. **Franklin, I.K.** *and C.B. Wollheim,* GABA in the endocrine pancreas: its putative role as an islet cell paracrine-signalling molecule. *J Gen Physiol, 2004.* ***123****(3): p. 185-90.*

90. **Frederikse, P.H.,** *et al.,* Synapsin and synaptic vesicle protein expression during embryonic and post-natal lens fiber cell differentiation. *Mol Vis, 2004.* ***10****: p. 794-804.*

91. **Frenzel, E.M.** *and R.G. Johnson,* Gap junction formation between cultured embryonic lens cells is inhibited by antibody to N-cadherin. *Dev Biol, 1996.* ***179****(1): p. 1-16.*

92. **Freund, T.F.** *and G. Buzsaki,* Interneurons of the hippocampus. *Hippocampus, 1996.* ***6****(4): p. 347-470.*

93. **Fritschy, J.M.,** *et al.,* GABAB-receptor splice variants GB1a and GB1b in rat brain: developmental regulation, cellular distribution and extrasynaptic localization. *Eur J Neurosci, 1999.* ***11****(3): p. 761-8.*

94. **Fritschy, J.M.** *and H. Mohler,* GABAA-receptor heterogeneity in the adult rat brain: differential regional and cellular distribution of seven major subunits. *J Comp Neurol, 1995.* ***359****(1): p. 154-94.*

95. **Fujii-Yamamoto, H.,** *et al.,* Cell cycle and developmental regulations of replication factors in mouse embryonic stem cells. *J Biol Chem, 2005.* ***280****(13): p. 12976-87.*

96. **Fukui, M.,** *et al.,* Modulation of cellular proliferation and differentiation through GABA(B) receptors expressed by undifferentiated neural progenitor cells isolated from fetal mouse brain. *J Cell Physiol, 2008.* ***216****(2): p. 507-19.*

97. **Furuta, Y.** *and B.L. Hogan,* BMP4 is essential for lens induction in the mouse embryo. *Genes Dev, 1998.* ***12****(23): p. 3764-75.*

98. **Gammelsaeter, R.,** *et al.,* Glycine, GABA and their transporters in pancreatic islets of Langerhans: evidence for a paracrine transmitter interplay. *J Cell Sci, 2004.* ***117****(Pt 17): p. 3749-58.*

99. **Garry, D.J.,** *M.G. Garry, and R.L. Sorenson,* Ultrastructural immunocytochemical localization of L-glutamate decarboxylase and GABA in rat pancreatic zymogen granules. *Cell Tissue Res, 1988.* ***252****(1): p. 191-7.*

100. **Garry, D.J.,** *R.L. Sorenson, and H.D. Coulter,* Ultrastructural localization of gamma amino

butyric acid immunoreactivity in B cells of the rat pancreas. *Diabetologia, 1987.* ***30****(2): p. 115-9.*

101. ***Garry, D.J.,*** *et al.,* Immunohistochemical colocalization of GABA and insulin in beta-cells of rat islet. *Diabetes, 1986.* ***35****(10): p. 1090-5.*

102. ***Geigerseder, C.,*** *et al.,* Evidence for a GABAergic system in rodent and human testis: local GABA production and GABA receptors. *Neuroendocrinology, 2003.* ***77****(5): p. 314-23.*

103. ***Gilon, P.,*** *et al.,* The influence of gamma-aminobutyric acid on hormone release by the mouse and rat endocrine pancreas. *Endocrinology, 1991.* ***129****(5): p. 2521-9.*

104. ***Gilon, P****. and C. Remacle,* High-affinity GABA uptake in a subpopulation of somatostatin cells in rat pancreas. *J Histochem Cytochem, 1989.* ***37****(7): p. 1133-9.*

105. ***Gilon, P.,*** *et al.,* G protein-dependent inhibition of L-type Ca2+ currents by acetylcholine in mouse pancreatic B-cells. *J Physiol, 1997.* ***499 (Pt 1)****: p. 65-76.*

106. ***Ginis, I.,*** *et al.,* Differences between human and mouse embryonic stem cells. *Dev Biol, 2004.* ***269****(2): p. 360-80.*

107. ***Gladkevich, A.,*** *et al.,* The peripheral GABAergic system as a target in endocrine disorders. *Auton Neurosci, 2006.* ***124****(1-2): p. 1-8.*

108. ***Glykys, J.,*** *et al.,* A new naturally occurring GABA(A) receptor subunit partnership with high sensitivity to ethanol. *Nat Neurosci, 2007.* ***10****(1): p. 40-8.*

109. ***Gottlieb, D.I.,*** Large-scale sources of neural stem cells. *Annu Rev Neurosci, 2002.* ***25****: p. 381-407.*

110. ***Guatteo, E.,*** *et al.,* Voltage-gated calcium channels mediate intracellular calcium increase in weaver dopaminergic neurons during stimulation of D2 and GABAB receptors. *J Neurophysiol, 2004.* ***92****(6): p. 3368-74.*

111. ***Hagiwara, N.,*** *et al.,* Nonneuronal expression of the GABA(A) beta3 subunit gene is required for normal palate development in mice. *Dev Biol, 2003.* ***254****(1): p. 93-101.*

112. ***Han, H.J****. and Y.J. Lee,* Insulin stimulates Ca2+ uptake via PKC, cAMP, and p38 MAPK in mouse embryonic stem cells. *Life Sci, 2005.* ***76****(25): p. 2903-19.*

113. ***Harding, C.V.,*** *et al.,* The control of cell division in the ocular lens. *Int Rev Cytol, 1971.* ***31****: p. 215-300.*

114. ***Hart, A.H.,*** *et al.,* Identification, cloning and expression analysis of the pluripotency promoting Nanog genes in mouse and human. *Dev Dyn, 2004.* ***230****(1): p. 187-98.*

115. ***Hashimoto, T****. and K. Kuriyama,* In vivo evidence that GABA(B) receptors are negatively coupled to adenylate cyclase in rat striatum. *J Neurochem, 1997.* ***69****(1): p. 365-70.*

116. ***Hawrot, E.,*** *et al.,* Demonstration of a tandem pair of complement protein modules in GABA(B) receptor 1a. *FEBS Lett, 1998.* ***432****(3): p. 103-8.*

117. ***Haydar, T.F.,*** *et al.,* Differential modulation of proliferation in the neocortical ventricular and subventricular zones. *J Neurosci, 2000.* ***20****(15): p. 5764-74.*

118. ***Hill, D.R.*** *and N.G. Bowery,* 3H-baclofen and 3H-GABA bind to bicuculline-insensitive

GABA B sites in rat brain. *Nature, 1981.* ***290****(5802): p. 149-52.*

119. ***Homanics, G.E.,*** *et al.,* Mice devoid of gamma-aminobutyrate type A receptor beta3 subunit have epilepsy, cleft palate, and hypersensitive behavior. *Proc Natl Acad Sci U S A, 1997.* ***94****(8): p. 4143-8.*

120. ***Homanics, G.E.,*** *et al.,* Normal electrophysiological and behavioral responses to ethanol in mice lacking the long splice variant of the gamma2 subunit of the gamma-aminobutyrate type A receptor. *Neuropharmacology, 1999.* ***38****(2): p. 253-65.*

121. ***Hu, J.H.,*** *X.B. He, and Y.C. Yan,* Identification of gamma-aminobutyric acid transporter (GAT1) on the rat sperm. *Cell Res, 2000.* ***10****(1): p. 51-8.*

122. ***Hubner, C.A.,*** *et al.,* Disruption of KCC2 reveals an essential role of K-Cl cotransport already in early synaptic inhibition. *Neuron, 2001.* ***30****(2): p. 515-24.*

123. ***Hyatt, G.A****. and D.C. Beebe,* Regulation of lens cell growth and polarity by an embryo-specific growth factor and by inhibitors of lens cell proliferation and differentiation. *Development, 1993.* ***117****(2): p. 701-9.*

124. ***Ishizaki, Y.,*** *M.D. Jacobson, and M.C. Raff,* A role for caspases in lens fiber differentiation. *J Cell Biol, 1998.* ***140****(1): p. 153-8.*

125. ***Jacob, T.C.,*** *S.J. Moss, and R. Jurd,* GABA(A) receptor trafficking and its role in the dynamic modulation of neuronal inhibition. *Nat Rev Neurosci, 2008.* ***9****(5): p. 331-43.*

126. ***Jean, D., K. Ewan****, and P. Gruss,* Molecular regulators involved in vertebrate eye development. *Mech Dev, 1998.* ***76****(1-2): p. 3-18.*

127. ***Jiang, Y.,*** *et al.,* Discovery of differentially expressed genes in human breast cancer using subtracted cDNA libraries and cDNA microarrays. *Oncogene, 2002.* ***21****(14): p. 2270-82.*

128. ***Jin, N.,*** *et al.,* Differential expression of GABAA receptor pi subunit in cultured rat alveolar epithelial cells. *Cell Tissue Res, 2005.* ***321****(2): p. 173-83.*

129. ***Jirmanova, L.,*** *et al.,* Differential contributions of ERK and PI3-kinase to the regulation of cyclin D1 expression and to the control of the G1/S transition in mouse embryonic stem cells. *Oncogene, 2002.* ***21****(36): p. 5515-28.*

130. ***Johnston, G.A.,*** GABAc receptors: relatively simple transmitter -gated ion channels? *Trends Pharmacol Sci, 1996.* ***17****(9): p. 319-23.*

131. ***Jones, K.A.,*** *et al.,* GABA(B) receptors function as a heteromeric assembly of the subunits GABA(B)R1 and GABA(B)R2. *Nature, 1998.* ***396****(6712): p. 674-9.*

132. ***Jursky, F.*** *and N. Nelson,* Developmental expression of GABA transporters GAT1 and GAT4 suggests involvement in brain maturation. *J Neurochem, 1996.* ***67****(2): p. 857-67.*

133. ***Jursky, F.*** *and N. Nelson,* Developmental expression of the neurotransmitter transporter GAT3. *J Neurosci Res, 1999.* ***55****(3): p. 394-9.*

134. ***Kaila, K.,*** Ionic basis of GABAA receptor channel function in the nervous system. *Prog Neurobiol, 1994.* ***42****(4): p. 489-537.*

135. ***Kaneda, M.,*** *M. Farrant, and S.G. Cull-Candy,* Whole-cell and single-channel currents

activated by GABA and glycine in granule cells of the rat cerebellum. *J Physiol, 1995.* ***485 (Pt 2)****: p. 419-35.*

136. ***Kania****, G., et al.,* Somatic stem cell marker prominin-1/CD133 is expressed in embryonic stem cell-derived progenitors. *Stem Cells, 2005.* ***23****(6): p. 791-804.*

137. ***Kapur,*** *N., G.A. Mignery, and K. Banach,* Cell cycle-dependent calcium oscillations in mouse embryonic stem cells. *Am J Physiol Cell Physiol, 2007.* ***292****(4): p. C1510-8.*

138. ***Karakossian,*** *M.H., et al.,* Novel properties of a mouse gamma-aminobutyric acid transporter (GAT4). *J Membr Biol, 2005.* ***203****(2): p. 65-82.*

139. ***Kasparov, S****., et al.,* GABA(A) receptor epsilon-subunit may confer benzodiazepine insensitivity to the caudal aspect of the nucleus tractus solitarii of the rat. *J Physiol, 2001.* ***536****(Pt 3): p. 785-96.*

140. ***Kataoka, Y.,*** *et al.,* Intrinsic GABAergic system of adrenal chromaffin cells. *Proc Natl Acad Sci U S A, 1984.* ***81****(10): p. 3218-22.*

141. ***Katarova, Z****., et al.,* Domain-restricted expression of two glutamic acid decarboxylase genes in midgestation mouse embryos. *J Comp Neurol, 2000.* ***424****(4): p. 607-27.*

142. ***Katarova, Z.,*** *et al.,* Molecular Identification of the 62 kd Form of Glutamic Acid Decarboxylase from the Mouse. *Eur J Neurosci, 1990.* ***2****(3): p. 190-202.*

143. ***Kaufman, D.L****., et al.,* Brain glutamate decarboxylase cloned in lambda gt-11: fusion protein produces gamma-aminobutyric acid. *Science, 1986.* ***232****(4754): p. 1138-40.*

144. ***Kaufman, M.H****.,* The atlas of mouse development. *1992, London ; San Diego: Academic Press. xvi, 512 p.*

145. ***Kaupmann, K.,*** *et al.,* Expression cloning of GABA(B) receptors uncovers similarity to metabotropic glutamate receptors. *Nature, 1997.* ***386****(6622): p. 239-46.*

146. ***Kawauchi, S.,*** *et al.,* Regulation of lens fiber cell differentiation by transcription factor c-Maf. *J Biol Chem, 1999.* ***274****(27): p. 19254-60.*

147. ***Kelsch, W.,*** *et al.,* Insulin-like growth factor 1 and a cytosolic tyrosine kinase activate chloride outward transport during maturation of hippocampal neurons. *J Neurosci, 2001.* ***21****(21): p. 8339-47.*

148. ***Kim, J.I.,*** *et al.,* Requirement for the c-Maf transcription factor in crystallin gene regulation and lens development. *Proc Natl Acad Sci U S A, 1999.* ***96****(7): p. 3781-5.*

149. ***Kleinrok, Z.,*** *et al.,* GABA content and GAD activity in colon tumors taken from patients with colon cancer or from xenografted human colon cancer cells growing as s.c. tumors in athymic nu/nu mice. *J Physiol Pharmacol, 1998.* ***49****(2): p. 303-10.*

150. ***Kobayashi, Y.,*** *D.L. Kaufman, and A.J. Tobin,* Glutamic acid decarboxylase cDNA: nucleotide sequence encoding an enzymatically active fusion protein. *J Neurosci, 1987.* ***7****(9): p. 2768-72.*

151. ***Kok, A.,*** *et al.,* Influence of platelet-derived growth factor on lens epithelial cell proliferation and differentiation. *Growth Factors, 2002.* ***20****(1): p. 27-34.*

152. **Kralic, J.E.,** *et al.,* Molecular and pharmacological characterization of GABA(A) receptor alpha1 subunit knockout mice. *J Pharmacol Exp Ther, 2002.* ***302****(3): p. 1037-45.*

153. **Kuroda, Y.,** *et al.,* Are there inositol 1,4,5-triphosphate (IP3) receptors in human sperm? *Life Sci, 1999.* ***65****(2): p. 135-43.*

154. **Kwakowsky, A.,** *et al.*, GABA neurotransmitter signaling in the developing mouse lens: dynamic regulation of components and functionality. *Dev Dyn, 2008.* ***237****(12): p. 3830-41.*

155. **Kwakowsky, A., et al.,** GAD isoforms exhibit distinct spatiotemporal expression patterns in the developing mouse lens: correlation with Dlx2 and Dlx5. *Dev Dyn, 2007.* ***236****(12): p. 3532-44.*

156. **Lancaster, G.A.,** *F. Mohyuddin, and C.R. Scriver,* Ontogeny of L-glutamic acid decarboxylase and gamma-aminobutyric acid concentration in human kidney. *Pediatr Res, 1975.* ***9****(5): p. 484-7.*

157. **Lauder, J.M.,** *et al.,* Prenatal ontogeny of the GABAergic system in the rat brain: an immunocytochemical study. *Neuroscience, 1986.* ***19****(2): p. 465-93.*

158. **Laurie, D.J.,** *W. Wisden, and P.H. Seeburg,* The distribution of thirteen GABAA receptor subunit mRNAs in the rat brain. III. Embryonic and postnatal development. *J Neurosci, 1992.* ***12****(11): p. 4151-72.*

159. **Leahy, A.,** *et al.,* Use of developmental marker genes to define temporal and spatial patterns of differentiation during embryoid body formation. *J Exp Zool, 1999.* ***284****(1): p. 67-81.*

160. **Lee, H.,** *et al.,* KCC2 expression in immature rat cortical neurons is sufficient to switch the polarity of GABA responses. *Eur J Neurosci, 2005.* ***21****(9): p. 2593-9.*

161. **Lee, M.Y.,** *J.S. Heo, and H.J. Han,* Dopamine regulates cell cycle regulatory proteins via cAMP, Ca(2+)/PKC, MAPKs, and NF-kappaB in mouse embryonic stem cells. *J Cell Physiol, 2006.* ***208****(2): p. 399-406.*

162. **Leventhal, A.G.,** *et al.*, GABA and its agonists improved visual cortical function in senescent monkeys. *Science, 2003.* ***300****(5620): p. 812-5.*

163. **Levi, G.,** *et al.,* Msx1 and Dlx5 act independently in development of craniofacial skeleton, but converge on the regulation of Bmp signaling in palate formation. *Mech Dev, 2006.* ***123****(1): p. 3-16.*

164. **Levi, G.** *and M. Raiteri,* Carrier-mediated release of neurotransmitters. *Trends Neurosci, 1993.* ***16****(10): p. 415-9.*

165. **Li, X.,** *et al.,* Transient expression of glutamate decarboxylase and gamma-amino butyric acid in embryonic lens fibers of the rat. *Dev Dyn, 1995.* ***203****(4): p. 448-55.*

166. **Ligon, B.,** *et al.*, Regulation of pancreatic islet cell survival and replication by gamma-aminobutyric acid. *Diabetologia, 2007.* ***50****(4): p. 764-73.*

167. **Liu, J.K.,** *et al.*, Dlx genes encode DNA-binding proteins that are expressed in an overlapping and sequential pattern during basal ganglia differentiation. *Dev Dyn, 1997.* ***210****(4): p. 498-512.*

168. **Liu, Q.R.,** *et al.*, Molecular characterization of four pharmacologically distinct gamma-aminobutyric acid transporters in mouse brain [corrected]. *J Biol Chem, 1993.* ***268****(3): p. 2106-12.*

169. **Liu, Z.F.** *and D.R. Burt,* A synthetic standard for competitive RT-PCR quantitation of 13 GABA receptor type A subunit mRNAs in rats and mice. *J Neurosci Methods, 1998.* ***85****(1): p. 89-98.*

170. **Lopez-Bendito, G.,** *et al.*, Blockade of GABA(B) receptors alters the tangential migration of cortical neurons. *Cereb Cortex, 2003.* ***13****(9): p. 932-42.*

171. **LoTurco, J.J**., *et al.*, GABA and glutamate depolarize cortical progenitor cells and inhibit DNA synthesis. *Neuron, 1995.* ***15****(6): p. 1287-98.*

172. **Louzan, P.,** *M.G. Gallardo, and J.H. Tramezzani,* Gamma-aminobutyric acid in the genital tract of the rat during the oestrous cycle. *J Reprod Fertil, 1986.* ***77****(2): p. 499-504.*

173. **Lovicu, F.J.** *and J.W. McAvoy,* Growth factor regulation of lens development. *Dev Biol, 2005.* ***280****(1): p. 1-14.*

174. **Lu, J., M.** *Karadsheh, and E. Delpire,* Developmental regulation of the neuronal-specific isoform of K-Cl cotransporter KCC2 in postnatal rat brains. *J Neurobiol, 1999.* ***39****(4): p. 558-68.*

175. **Lujan, R.,** *R. Shigemoto, and G. Lopez-Bendito,* Glutamate and GABA receptor signalling in the developing brain. *Neuroscience, 2005.* ***130****(3): p. 567-80.*

176. **Lukasiewicz, P.D.,** GABAC receptors in the vertebrate retina. *Mol Neurobiol, 1996.* ***12****(3): p. 181-94.*

177. **Ma, W.,** *et al.*, Transient increase in expression of GAD65 and GAD67 mRNAs during postnatal development of rat spinal cord. *J Comp Neurol, 1994.* ***346****(1): p. 151-60.*

178. **Ma, W.,** *et al.*, Ontogeny of GABAA receptor subunit mRNAs in rat spinal cord and dorsal root ganglia. *J Comp Neurol, 1993.* ***338****(3): p. 337-59.*

179. **Ma, Y.H.,** *et al.*, Gamma-aminobutyric acid transporter (GAT1) overexpression in mouse affects the testicular morphology. *Cell Res, 2000.* ***10****(1): p. 59-69.*

180. **Maddox, D.M.** *and B.G. Condie,* Dynamic expression of a glutamate decarboxylase gene in multiple non-neural tissues during mouse development. *BMC Dev Biol, 2001.* ***1****: p. 1.*

181. **Maemura, K.,** *et al.*, Gamma-amino-butyric acid immunoreactivity in intramucosal colonic tumors. *J Gastroenterol Hepatol, 2003.* ***18****(9): p. 1089-94.*

182. **Magnaghi, V.,** *et al.*, GABAB receptors in Schwann cells influence proliferation and myelin protein expression. *Eur J Neurosci, 2004.* ***19****(10): p. 2641-9.*

183. **Manent, J.B.**, *et al.*, A noncanonical release of GABA and glutamate modulates neuronal migration. *J Neurosci, 2005.* ***25****(19): p. 4755-65.*

184. **Manent, J.B.** *and A. Represa,* Neurotransmitters and brain maturation: early paracrine actions of GABA and glutamate modulate neuronal migration. *Neuroscientist, 2007.* ***13****(3): p. 268-79.*

185. ***Marcus, M.*** *and Y.S. Halpern,* Genetic and physiological analysis of glutamate decarboxylase in Escherichia coli K-12. *J Bacteriol, 1969.* ***97****(3): p. 1509-10.*

186. ***Maric, D.,*** *et al.,* GABA expression dominates neuronal lineage progression in the embryonic rat neocortex and facilitates neurite outgrowth via GABA(A) autoreceptor/Cl- channels. *J Neurosci, 2001.* ***21****(7): p. 2343-60.*

187. ***Martin, D.L.*** *and K.E. Barke,* Are GAD65 and GAD67 associated with specific pools of GABA in brain? *Perspect Dev Neurobiol, 1998.* ***5****(2-3): p. 119-29.*

188. ***Martin, D.L.,*** *et al.,* Structural features and regulatory properties of the brain glutamate decarboxylases. *Neurochem Int, 2000.* ***37****(2-3): p. 111-9.*

189. ***Martin, D.L.****, et al.,* Regulatory properties of brain glutamate decarboxylase (GAD): the apoenzyme of GAD is present principally as the smaller of two molecular forms of GAD in brain. *J Neurosci, 1991.* ***11****(9): p. 2725-31.*

190. ***Martin, D.L.*** *and K. Rimvall,* Regulation of gamma-aminobutyric acid synthesis in the brain. *J Neurochem, 1993.* ***60****(2): p. 395-407.*

191. ***Martin, G.R.,*** Isolation of a pluripotent cell line from early mouse embryos cultured in medium conditioned by teratocarcinoma stem cells. *Proc Natl Acad Sci U S A, 1981.* ***78****(12): p. 7634-8.*

192. ***Martin, S.C.****, et al.,* Differential expression of gamma-aminobutyric acid type B receptor subunit mRNAs in the developing nervous system and receptor coupling to adenylyl cyclase in embryonic neurons. *J Comp Neurol, 2004.* ***473****(1): p. 16-29.*

193. ***Marty, S.,*** *et al.,* GABAergic stimulation regulates the phenotype of hippocampal interneurons through the regulation of brain-derived neurotrophic factor. *Neuron, 1996.* ***16****(3): p. 565-70.*

194. ***Matsuda, T.,*** *et al.,* STAT3 activation is sufficient to maintain an undifferentiated state of mouse embryonic stem cells. *Embo J, 1999.* ***18****(15): p. 4261-9.*

195. ***Matuszek, M.,*** *M. Jesipowicz, and Z. Kleinrok,* GABA content and GAD activity in gastric cancer. *Med Sci Monit, 2001. 7(3): p. 377-81.*

196. ***McAvoy, J.W.,*** *et al.,* Lens development. *Eye, 1999.* ***13 (Pt 3b)****: p. 425-37.*

197. ***McIntire, S.L.,*** *et al.,* Identification and characterization of the vesicular GABA transporter. *Nature, 1997.* ***389****(6653): p. 870-6.*

198. ***Medina-Kauwe, L.K.,*** *et al.,* 4-Aminobutyrate aminotransferase (GABA-transaminase) deficiency. *J Inherit Metab Dis, 1999.* ***22****(4): p. 414-27.*

199. ***Meier, J.*** *and R. Grantyn,* A gephyrin-related mechanism restraining glycine receptor anchoring at GABAergic synapses. *J Neurosci, 2004.* ***24****(6): p. 1398-405.*

200. ***Meier, S.D.,*** *K.W. Kafitz, and C.R. Rose,* Developmental profile and mechanisms of GABA-induced calcium signaling in hippocampal astrocytes. *Glia, 2008.* ***56****(10): p. 1127-37.*

201. ***Menko, A.S.****, K.A. Klukas, and R.G. Johnson,* Chicken embryo lens cultures mimic differentiation in the lens. *Dev Biol, 1984.* ***103****(1): p. 129-41.*

202. **Merlo, G.R.,** *et al.*, Multiple functions of Dlx genes. *Int J Dev Biol, 2000.* ***44****(6): p. 619-26.*

203. **Mihalek, R.M.,** *et al.*, Attenuated sensitivity to neuroactive steroids in gamma-aminobutyrate type A receptor delta subunit knockout mice. *Proc Natl Acad Sci U S A, 1999.* ***96****(22): p. 12905-10.*

204. **Mintz, E.M.,** *et al.*, GABA interacts with photic signaling in the suprachiasmatic nucleus to regulate circadian phase shifts. *Neuroscience, 2002.* ***109****(4): p. 773-8.*

205. **Mintz, I.M.** *and B.P. Bean,* GABAB receptor inhibition of P-type Ca2+ channels in central neurons. *Neuron, 1993.* ***10****(5): p. 889-98.*

206. **Mitsui, K.,** *et al.*, The homeoprotein Nanog is required for maintenance of pluripotency in mouse epiblast and ES cells. *Cell, 2003.* ***113****(5): p. 631-42.*

207. **Mizuta, K.,** *et al.*, Functional expression of GABAB receptors in airway epithelium. *Am J Respir Cell Mol Biol, 2008.* ***39****(3): p. 296-304.*

208. **Mizuta, K.,** *et al.*, GABAA receptors are expressed and facilitate relaxation in airway smooth muscle. *Am J Physiol Lung Cell Mol Physiol, 2008.* ***294****(6): p. L1206-16.*

209. **Mody, I.,** Extrasynaptic GABAA receptors in the crosshairs of hormones and ethanol. *Neurochem Int, 2008.* ***52****(1-2): p. 60-4.*

210. **Mohler, H.,** GABA(A) receptor diversity and pharmacology. *Cell Tissue Res, 2006.* ***326****(2): p. 505-16.*

211. **Mombereau, C.,** *et al.*, Altered response to benzodiazepine anxiolytics in mice lacking GABA B(1) receptors. *Eur J Pharmacol, 2004.* ***497****(1): p. 119-20.*

212. **Muth, T.R.,** *J. Ahn, and M.J. Caplan,* Identification of sorting determinants in the C-terminal cytoplasmic tails of the gamma-aminobutyric acid transporters GAT-2 and GAT-3. *J Biol Chem, 1998.* ***273****(40): p. 25616-27.*

213. **Nagy, A.,** *et al.*, Derivation of completely cell culture-derived mice from early-passage embryonic stem cells. *Proc Natl Acad Sci U S A, 1993.* ***90****(18): p. 8424-8.*

214. **Neelands, T.R.** *and R.L. Macdonald,* Incorporation of the pi subunit into functional gamma-aminobutyric Acid(A) receptors. *Mol Pharmacol, 1999.* ***56****(3): p. 598-610.*

215. **New, D.C.,** *et al.*, GABAB heterodimeric receptors promote Ca2+ influx via store-operated channels in rat cortical neurons and transfected Chinese hamster ovary cells. *Neuroscience, 2006.* ***137****(4): p. 1347-58.*

216. **Nichols, J.,** *et al.*, Formation of pluripotent stem cells in the mammalian embryo depends on the POU transcription factor Oct4. *Cell, 1998.* ***95****(3): p. 379-91.*

217. **Nicoll, R.A.,** My close encounter with GABA(B) receptors. *Biochem Pharmacol, 2004.* ***68****(8): p. 1667-74.*

218. **Niwa, H.,** [Self-renewal and differentiation of ES cells]. *Hum Cell, 2000.* ***13****(4): p. 161-75.*

219. **Niwa, H.,** Molecular mechanism to maintain stem cell renewal of ES cells. *Cell Struct Funct, 2001.* ***26****(3): p. 137-48.*

220. **Niwa, H.,** How is pluripotency determined and maintained? *Development, 2007.* ***134****(4): p.*

635-46.

221. ***Niwa, H.,*** *et al.,* Self-renewal of pluripotent embryonic stem cells is mediated via activation of STAT3. *Genes Dev, 1998.* ***12****(13): p. 2048-60.*

222. ***Nusser, Z.*** *and I. Mody,* Selective modulation of tonic and phasic inhibitions in dentate gyrus granule cells. *J Neurophysiol, 2002.* ***87****(5): p. 2624-8.*

223. ***Odagaki, Y.*** *and T. Koyama,* Identification of galpha subtype(s) involved in gamma-aminobutyric acid(B) receptor-mediated high-affinity guanosine triphosphatase activity in rat cerebral cortical membranes. *Neurosci Lett, 2001.* ***297****(2): p. 137-41.*

224. ***Ogino, H.*** *and K. Yasuda,* Sequential activation of transcription factors in lens induction. *Dev Growth Differ, 2000.* ***42****(5): p. 437-48.*

225. ***Oh, W.J.,*** *et al.,* The mouse vesicular inhibitory amino acid transporter gene: expression during embryogenesis, analysis of its core promoter in neural stem cells and a reconsideration of its alternate splicing. *Gene, 2005.* ***351****: p. 39-49.*

226. ***Okada, M.,*** *et al.,* Functional correlation of GABA(A) receptor alpha subunits expression with the properties of IPSCs in the developing thalamus. *J Neurosci, 2000.* ***20****(6): p. 2202-8.*

227. ***Ong, J.*** *and D.I. Kerr,* Recent advances in GABAB receptors: from pharmacology to molecular biology. *Acta Pharmacol Sin, 2000.* ***21****(2): p. 111-23.*

228. ***Opolski, A.,*** *et al.,* The role of GABA-ergic system in human mammary gland pathology and in growth of transplantable murine mammary cancer. *J Exp Clin Cancer Res, 2000.* ***19****(3): p. 383-90.*

229. ***Oppitz, M.,*** *A. Mack, and U. Drews,* Ca2+-mobilization and cell contraction after muscarinic cholinergic stimulation of the chick embryo lens. *Invest Ophthalmol Vis Sci, 2003.* ***44****(11): p. 4813-9.*

230. ***Owens, D.F.,*** *et al.,* Excitatory GABA responses in embryonic and neonatal cortical slices demonstrated by gramicidin perforated-patch recordings and calcium imaging. *J Neurosci, 1996.* ***16****(20): p. 6414-23.*

231. ***Owens, D.F.*** *and A.R. Kriegstein,* Patterns of intracellular calcium fluctuation in precursor cells of the neocortical ventricular zone. *J Neurosci, 1998.* ***18****(14): p. 5374-88.*

232. ***Owens, D.F.*** *and A.R. Kriegstein,* Is there more to GABA than synaptic inhibition? *Nat Rev Neurosci, 2002.* ***3****(9): p. 715-27.*

233. ***Owens, D.F.*** *and A.R. Kriegstein,* Developmental neurotransmitters? *Neuron, 2002.* ***36****(6): p. 989-91.*

234. ***Owens, D.F.,*** *X. Liu, and A.R. Kriegstein,* Changing properties of GABA(A) receptor-mediated signaling during early neocortical development. *J Neurophysiol, 1999.* ***82****(2): p. 570-83.*

235. ***Panganiban, G****. and J.L. Rubenstein,* Developmental functions of the Distal-less/Dlx homeobox genes. *Development, 2002.* ***129****(19): p. 4371-86.*

236. ***Parducz, A.,*** *et al.,* GABA-immunoreactive structures in rat kidney. *J Histochem Cytochem,*

*1992. **40**(5): p. 675-80.*

237. ***Parra, P.,** A.I. Gulyas, and R. Miles,* How many subtypes of inhibitory cells in the hippocampus? *Neuron, 1998. **20**(5): p. 983-93.*

238. ***Parramon, M.,** et al.,* GABAB receptors increase intracellular calcium concentrations in chromaffin cells through two different pathways: their role in catecholamine secretion. *J Neurosci Res, 1995. **41**(1): p. 65-72.*

239. ***Paulsen, O.** and E.I. Moser,* A model of hippocampal memory encoding and retrieval: GABAergic control of synaptic plasticity. *Trends Neurosci, 1998. **21**(7): p. 273-8.*

240. ***Payne, J.A.,*** Functional characterization of the neuronal-specific K-Cl cotransporter: implications for [K+]o regulation. *Am J Physiol, 1997. **273**(5 Pt 1): p. C1516-25.*

241. ***Payne, J.A**., T.J. Stevenson, and L.F. Donaldson,* Molecular characterization of a putative K-Cl cotransporter in rat brain. A neuronal-specific isoform. *J Biol Chem, 1996. **271**(27): p. 16245-52.*

242. ***Pearson, R.,** et al.,* Purinergic and muscarinic modulation of the cell cycle and calcium signaling in the chick retinal ventricular zone. *J Neurosci, 2002. **22**(17): p. 7569-79.*

243. ***Peng, Z.,** et al.,* Altered expression of the delta subunit of the GABAA receptor in a mouse model of temporal lobe epilepsy. *J Neurosci, 2004. **24**(39): p. 8629-39.*

244. ***Pesce, M.** and H.R. Scholer,* Oct-4: gatekeeper in the beginnings of mammalian development. *Stem Cells, 2001. **19**(4): p. 271-8.*

245. ***Pierson, R.C.,** A.M. Lyons, and L.J. Greenfield, Jr.,* Gonadal steroids regulate GABAA receptor subunit mRNA expression in NT2-N neurons. *Brain Res Mol Brain Res, 2005. **138**(2): p. 105-15.*

246. ***Pin, J.P.** and J. Bockaert,* Two distinct mechanisms, differentially affected by excitatory amino acids, trigger GABA release from fetal mouse striatal neurons in primary culture. *J Neurosci, 1989. **9**(2): p. 648-56.*

247. ***Plummer, H.K**., 3rd, et al.,* Expression of inwardly rectifying potassium channels (GIRKs) and beta-adrenergic regulation of breast cancer cell lines. *BMC Cancer, 2004. **4**: p. 93.*

248. ***Poncer, J.C.,** et al.,* Either N- or P-type calcium channels mediate GABA release at distinct hippocampal inhibitory synapses. *Neuron, 1997. **18**(3): p. 463-72.*

249. ***Porteus, M.H.**, et al.,* DLX-2, MASH-1, and MAP-2 expression and bromodeoxyuridine incorporation define molecularly distinct cell populations in the embryonic mouse forebrain. *J Neurosci, 1994. **14**(11 Pt 1): p. 6370-83.*

250. ***Purcell, P.,** et al.,* Pax6-dependence of Six3, Eya1 and Dach1 expression during lens and nasal placode induction. *Gene Expr Patterns, 2005. **6**(1): p. 110-8.*

251. ***Racagni, G.,** et al.,* The hypothalamo-pituitary GABAergic regulation of prolactin release in the rat. *Adv Biochem Psychopharmacol, 1986. **42**: p. 103-17.*

252. ***Rafferty, N.S.** and K.A. Rafferty, Jr.,* Cell population kinetics of the mouse lens epithelium. *J Cell Physiol, 1981. **107**(3): p. 309-15.*

253. **Rakic, P.,** Principles of neural cell migration. *Experientia, 1990.* ***46****(9): p. 882-91.*

254. **Ramadan, E.,** *et al.,* GABA(A) receptor beta3 subunit deletion decreases alpha2/3 subunits and IPSC duration. *J Neurophysiol, 2003.* ***89****(1): p. 128-34.*

255. **Reetz, A.,** *et al.,* GABA and pancreatic beta-cells: colocalization of glutamic acid decarboxylase (GAD) and GABA with synaptic-like microvesicles suggests their role in GABA storage and secretion. *Embo J, 1991.* ***10****(5): p. 1275-84.*

256. **Represa, A.** *and Y. Ben-Ari,* Trophic actions of GABA on neuronal development. *Trends Neurosci, 2005.* ***28****(6): p. 278-83.*

257. **Ring, B.Z.,** *et al.,* Regulation of mouse lens fiber cell development and differentiation by the Maf gene. *Development, 2000.* ***127****(2): p. 307-17.*

258. **Ritter, B.,** *et al.,* Subcellular vesicular aggregations of GABAB R1a and R1b receptors increase with age in neurons of the developing mouse brain. *Cell Tissue Res, 2005.* ***319****(2): p. 181-9.*

259. **Rivera, C.,** *et al.,* The K+/Cl- co-transporter KCC2 renders GABA hyperpolarizing during neuronal maturation. *Nature, 1999.* ***397****(6716): p. 251-5.*

260. **Roberts, E.,** Disinhibition as an organizing principle in the nervous system. The role of gamma-aminobutyric acid. *Adv Neurol, 1974.* ***5****: p. 127-43.*

261. **Roberts, E.,** GABA-related phenomena, models of nervous system function, and seizures. *Ann Neurol, 1984.* ***16 Suppl****: p. S77-89.*

262. **Roberts, S.S.,** *et al.,* GABAergic system gene expression predicts clinical outcome in patients with neuroblastoma. *J Clin Oncol, 2004.* ***22****(20): p. 4127-34.*

263. **Robinson, M.L.,** An essential role for FGF receptor signaling in lens development. *Semin Cell Dev Biol, 2006.* ***17****(6): p. 726-40.*

264. **Robinson, M.L.,** *et al.,* Expression of a truncated FGF receptor results in defective lens development in transgenic mice. *Development, 1995.* ***121****(12): p. 3959-67.*

265. **Robinson, M.L.,** *et al.,* Extracellular FGF-1 acts as a lens differentiation factor in transgenic mice. *Development, 1995.* ***121****(2): p. 505-14.*

266. **Rosemblit, N.,** *et al.,* Intracellular calcium release channel expression during embryogenesis. *Dev Biol, 1999.* ***206****(2): p. 163-77.*

267. **Rossi, D.** *and V. Sorrentino,* Molecular genetics of ryanodine receptors Ca2+-release channels. *Cell Calcium, 2002.* ***32****(5-6): p. 307-19.*

268. **Sakaba, T.** *and E. Neher,* Direct modulation of synaptic vesicle priming by GABA(B) receptor activation at a glutamatergic synapse. *Nature, 2003.* ***424****(6950): p. 775-8.*

269. **Sato, N.,** *et al.,* Maintenance of pluripotency in human and mouse embryonic stem cells through activation of Wnt signaling by a pharmacological GSK-3-specific inhibitor. *Nat Med, 2004.* ***10****(1): p. 55-63.*

270. **Schousboe, A.** *and D.A. Redburn,* Modulatory actions of gamma aminobutyric acid (GABA) on GABA type A receptor subunit expression and function. *J Neurosci Res, 1995.* ***41****(1): p.*

1-7.

271. ***Schousboe, A.,*** *et al.,* GABA transporters as drug targets for modulation of GABAergic activity. *Biochem Pharmacol, 2004.* ***68****(8): p. 1557-63.*

272. ***Shibata, S.,*** *et al.,* Experience-dependent changes in intracellular Cl- regulation in developing auditory neurons. *Neurosci Res, 2004.* ***48****(2): p. 211-20.*

273. ***Sieghart, W.*** *and G. Sperk,* Subunit composition, distribution and function of GABA(A) receptor subtypes. *Curr Top Med Chem, 2002.* ***2****(8): p. 795-816.*

274. ***Sim, J.A.,*** *et al.,* Late postnatal reorganization of GABA(A) receptor signalling in native GnRH neurons. *Eur J Neurosci, 2000.* ***12****(10): p. 3497-504.*

275. ***Simeone, A.*** *, et al.,* Cloning and characterization of two members of the vertebrate Dlx gene family. *Proc Natl Acad Sci U S A, 1994.* ***91****(6): p. 2250-4.*

276. ***Simon, J.,*** *et al.,* Analysis of the set of GABA(A) receptor genes in the human genome. *J Biol Chem, 2004.* ***279****(40): p. 41422-35.*

277. ***Simonds, W.F.,*** G protein regulation of adenylate cyclase. *Trends Pharmacol Sci, 1999.* ***20****(2): p. 66-73.*

278. ***Soghomonian, J.J****. and D.L. Martin,* Two isoforms of glutamate decarboxylase: why? *Trends Pharmacol Sci, 1998.* ***19****(12): p. 500-5.*

279. ***Solimena, M.,*** *et al.,* Association of GAD-65, but not of GAD-67, with the Golgi complex of transfected Chinese hamster ovary cells mediated by the N-terminal region. *Proc Natl Acad Sci U S A, 1993.* ***90****(7): p. 3073-7.*

280. ***Solter, D.,*** From teratocarcinomas to embryonic stem cells and beyond: a history of embryonic stem cell research. *Nat Rev Genet, 2006.* ***7****(4): p. 319-27.*

281. ***Somogyi, R****., et al.,* Developmental kinetics of GAD family mRNAs parallel neurogenesis in the rat spinal cord. *J Neurosci, 1995.* ***15****(4): p. 2575-91.*

282. ***Stewart, C.L.,*** *et al.,* Blastocyst implantation depends on maternal expression of leukaemia inhibitory factor. *Nature, 1992.* ***359****(6390): p. 76-9.*

283. ***Stock, D.W.,*** *et al.,* The evolution of the vertebrate Dlx gene family. *Proc Natl Acad Sci U S A, 1996.* ***93****(20): p. 10858-63.*

284. ***Stuhmer, T.,*** *et al.,* Ectopic expression of the Dlx genes induces glutamic acid decarboxylase and Dlx expression. *Development, 2002.* ***129****(1): p. 245-52.*

285. ***Stuhmer, T.,*** *et al.,* Expression from a Dlx gene enhancer marks adult mouse cortical GABAergic neurons. *Cereb Cortex, 2002.* ***12****(1): p. 75-85.*

286. ***Sun, S.,*** *et al.,* Physical manipulation of calcium oscillations facilitates osteodifferentiation of human mesenchymal stem cells. *Faseb J, 2007.* ***21****(7): p. 1472-80.*

287. ***Szabo, G.,*** *et al.,* Differential regulation of adult and embryonic glutamate decarboxylases in rat dentate granule cells after kainate-induced limbic seizures. *Neuroscience, 2000.* ***100****(2): p. 287-95.*

288. ***Szabo, G.,*** *Z. Katarova, and R. Greenspan,* Distinct protein forms are produced from

alternatively spliced bicistronic glutamic acid decarboxylase mRNAs during development. *Mol Cell Biol, 1994.* ***14****(11): p. 7535-45.*

289. ***Tamayama, T.,*** *et al.,* Expression of GABA(A) and GABA(B) receptors in rat growth plate chondrocytes: activation of the GABA receptors promotes proliferation of mouse chondrogenic ATDC5 cells. *Mol Cell Biochem, 2005.* ***273****(1-2): p. 117-26.*

290. ***Taylor, J.,*** *M. Docherty, and P.R. Gordon-Weeks,* GABAergic growth cones: release of endogenous gamma-aminobutyric acid precedes the expression of synaptic vesicle antigens. *J Neurochem, 1990.* ***54****(5): p. 1689-99.*

291. ***Taylor, J.*** *and P.R. Gordon-Weeks,* Calcium-independent gamma-aminobutyric acid release from growth cones: role of gamma-aminobutyric acid transport. *J Neurochem, 1991.* ***56****(1): p. 273-80.*

292. ***TenBroek, E.M.,*** *R. Johnson, and C.F. Louis,* Cell-to-cell communication in a differentiating ovine lens culture system. *Invest Ophthalmol Vis Sci, 1994.* ***35****(1): p. 215-28.*

293. ***Terasawa, E.,*** Role of GABA in the mechanism of the onset of puberty in non-human primates. *Int Rev Neurobiol, 2005.* ***71****: p. 113-29.*

294. ***Thomas, B.L.,*** *et al.,* Role of Dlx-1 and Dlx-2 genes in patterning of the murine dentition. *Development, 1997.* ***124****(23): p. 4811-8.*

295. ***Thomson, J.A.*** *and V.S. Marshall,* Primate embryonic stem cells. *Curr Top Dev Biol, 1998.* ***38****: p. 133-65.*

296. ***Thuault, S.J.,*** *et al.,* The GABA(B2) subunit is critical for the trafficking and function of native GABA(B) receptors. *Biochem Pharmacol, 2004.* ***68****(8): p. 1655-66.*

297. ***Tillakaratne, N.J.,*** *et al.,* Glutamate decarboxylases in nonneural cells of rat testis and oviduct: differential expression of GAD65 and GAD67. *J Neurochem, 1992.* ***58****(2): p. 618-27.*

298. ***Todorova, M.G.,*** *B. Soria, and I. Quesada,* Gap junctional intercellular communication is required to maintain embryonic stem cells in a non-differentiated and proliferative state. *J Cell Physiol, 2008.* ***214****(2): p. 354-62.*

299. ***Togel, M.,*** *et al.,* gamma-Aminobutyric acidA receptors displaying association of gamma 3-subunits with beta 2/3 and different alpha-subunits exhibit unique pharmacological properties. *J Biol Chem, 1994.* ***269****(17): p. 12993-8.*

300. ***Turner, J.D.,*** *et al.,* Immunohistochemical mapping of gamma-aminobutyric acid type-A receptor alpha subunits in rat central nervous system. *Psychopharmacol Ser, 1993.* ***11****: p. 29-49.*

301. ***Uhl, G.R.,*** Neurotransmitter transporters (plus): a promising new gene family. *Trends Neurosci, 1992.* ***15****(7): p. 265-8.*

302. ***Uusi-Oukari, M.,*** *et al.,* Long-range interactions in neuronal gene expression: evidence from gene targeting in the GABA(A) receptor beta2-alpha6-alpha1-gamma2 subunit gene cluster. *Mol Cell Neurosci, 2000.* ***16****(1): p. 34-41.*

303. **Varju, P.,** *et al.*, GABA signalling during development: new data and old questions. *Cell Tissue Res, 2001.* ***305****(2): p. 239-46.*

304. **Varju, P.,** *et al.*, Sequential induction of embryonic and adult forms of glutamic acid decarboxylase during in vitro-induced neurogenesis in cloned neuroectodermal cell-line, NE-7C2. *J Neurochem, 2002.* ***80****(4): p. 605-15.*

305. **Verkhratsky, A.,** Physiology and pathophysiology of the calcium store in the endoplasmic reticulum of neurons. *Physiol Rev, 2005.* ***85****(1): p. 201-79.*

306. **Verkhratsky, A.,** *R.K. Orkand, and H. Kettenmann,* Glial calcium: homeostasis and signaling function. *Physiol Rev, 1998.* ***78****(1): p. 99-141.*

307. **Verney, C.,** Phenotypic expression of monoamines and GABA in the early development of human telencephalon, transient or not transient. *J Chem Neuroanat, 2003.* ***26****(4): p. 283-92.*

308. **Vigot, R.,** *et al.*, Differential compartmentalization and distinct functions of GABAB receptor variants. *Neuron, 2006.* ***50****(4): p. 589-601.*

309. **Voutsinos, B.,** *et al.*, Serotoninergic control of the activity and expression of glial GABA transporters in the rat cerebellum. *Glia, 1998.* ***23****(1): p. 45-60.*

310. **Wagner, L.M***. and D.J. Takemoto,* Protein kinase C alpha and gamma in N/N 1003A rabbit lens epithelial cell differentiation. *Mol Vis, 2001.* ***7****: p. 57-62.*

311. **Wang, C.,** *Z. Ling, and D. Pipeleers,* Comparison of cellular and medium insulin and GABA content as markers for living beta-cells. *Am J Physiol Endocrinol Metab, 2005.* ***288****(2): p. E307-13.*

312. **Watanabe, M.,** *et al.*, GABA and GABA receptors in the central nervous system and other organs. *Int Rev Cytol, 2002.* ***213****: p. 1-47.*

313. **Watanabe, M.,** *et al.*, Gamma-aminobutyric acid (GABA) and cell proliferation: focus on cancer cells. *Histol Histopathol, 2006.* ***21****(10): p. 1135-41.*

314. **Wawersik, S.,** *et al.*, BMP7 acts in murine lens placode development. *Dev Biol, 1999.* ***207****(1): p. 176-88.*

315. **Webb, S.E.** *and A.L. Miller,* Calcium signalling during embryonic development. *Nat Rev Mol Cell Biol, 2003.* ***4****(7): p. 539-51.*

316. **Weber, G.F.** *and A.S. Menko,* The canonical intrinsic mitochondrial death pathway has a non-apoptotic role in signaling lens cell differentiation. *J Biol Chem, 2005.* ***280****(23): p. 22135-45.*

317. **Westmoreland, J.J.,** *C.R. Hancock, and B.G. Condie,* Neuronal development of embryonic stem cells: a model of GABAergic neuron differentiation. *Biochem Biophys Res Commun, 2001.* ***284****(3): p. 674-80.*

318. **Williams, M.R.,** *et al.*, Role of the endoplasmic reticulum in shaping calcium dynamics in human lens cells. *Invest Ophthalmol Vis Sci, 2001.* ***42****(5): p. 1009-17.*

319. **Wobus, A.M.** *and K.R. Boheler,* Embryonic stem cells: prospects for developmental biology and cell therapy. *Physiol Rev, 2005.* ***85****(2): p. 635-78.*

320. **Wolf, P.,** *et al.*, GABAergic inhibition of neurons in the ventral tegmental area. *Experientia,*

1978. ***34****(1): p. 73-4.*

321. ***Wolf, R.,*** *U. Tscherne, and H.M. Emrich,* Suppression of preoptic GABA release caused by push-pull-perfusion with sodium valproate. *Naunyn Schmiedebergs Arch Pharmacol, 1988.* ***338****(6): p. 658-63.*

322. ***Wong, C.G.,*** *T. Bottiglieri, and O.C. Snead, 3rd,* GABA, gamma-hydroxybutyric acid, and neurological disease. *Ann Neurol, 2003.* ***54 Suppl 6****: p. S3-12.*

323. ***Woo, N.S.,*** *et al.,* Hyperexcitability and epilepsy associated with disruption of the mouse neuronal-specific K-Cl cotransporter gene. *Hippocampus, 2002.* ***12****(2): p. 258-68.*

324. ***Woodin, M.A.,*** *K. Ganguly, and M.M. Poo,* Coincident pre- and postsynaptic activity modifies GABAergic synapses by postsynaptic changes in Cl- transporter activity. *Neuron, 2003.* ***39****(5): p. 807-20.*

325. ***Work, E.*** *and D.L. Dewey,* The distribution of alpha, epsilon-diaminopimelic acid among various micro-organisms. *J Gen Microbiol, 1953.* ***9****(3): p. 394-406.*

326. ***Wride, M.A.,*** Minireview: apoptosis as seen through a lens. *Apoptosis, 2000.* ***5****(3): p. 203-9.*

327. ***Wride, M.A.,*** *et al.,* Expression profiling and gene discovery in the mouse lens. *Mol Vis, 2003.* ***9****: p. 360-96.*

328. ***Wu, Y.,*** *et al.,* Nonvesicular inhibitory neurotransmission via reversal of the GABA transporter GAT-1. *Neuron, 2007.* ***56****(5): p. 851-65.*

329. ***Wunderlich, K.*** *and M. Knorr,* [Effect of platelet-derived growth factor PDGF on replication of cultivated bovine lens epithelial cells]. *Ophthalmologe, 1994.* ***91****(1): p. 98-102.*

330. ***Wyborski, R.J.,*** *R.W. Bond, and D.I. Gottlieb,* Characterization of a cDNA coding for rat glutamic acid decarboxylase. *Brain Res Mol Brain Res, 1990.* ***8****(3): p. 193-8.*

331. ***Xiang, Y.Y.,*** *et al.,* A GABAergic system in airway epithelium is essential for mucus overproduction in asthma. *Nat Med, 2007.* ***13****(7): p. 862-7.*

332. ***Xie, Z.,*** *K.P. Currie, and A.P. Fox,* Etomidate elevates intracellular calcium levels and promotes catecholamine secretion in bovine chromaffin cells. *J Physiol, 2004.* ***560****(Pt 3): p. 677-90.*

333. ***Yanagida, E.,*** *et al.,* Functional expression of Ca2+ signaling pathways in mouse embryonic stem cells. *Cell Calcium, 2004.* ***36****(2): p. 135-46.*

334. ***Ying, Q.L.,*** *et al.,* BMP induction of Id proteins suppresses differentiation and sustains embryonic stem cell self-renewal in collaboration with STAT3. *Cell, 2003.* ***115****(3): p. 281-92.*

335. ***Yu, H.M.,*** *et al.,* Critical role of type 2 ryanodine receptor in mediating activity-dependent neurogenesis from embryonic stem cells. *Cell Calcium, 2008.* ***43****(5): p. 417-31.*

336. ***Zandy, A.J.,*** *et al.,* Role of the executioner caspases during lens development. *J Biol Chem, 2005.* ***280****(34): p. 30263-72.*

337. ***Zecevic, N.*** *and A. Milosevic,* Initial development of gamma-aminobutyric acid immunoreactivity in the human cerebral cortex. *J Comp Neurol, 1997.* ***380****(4): p. 495-506.*

338. ***Zerucha, T.*** *and M. Ekker,* Distal-less-related homeobox genes of vertebrates: evolution, function, and regulation. *Biochem Cell Biol, 2000.* ***78****(5): p. 593-601.*

339. ***Zhang, D.****, et al.,* Structure and function of GABA(C) receptors: a comparison of native versus recombinant receptors. *Trends Pharmacol Sci, 2001.* ***22****(3): p. 121-32.*

340. ***Zhang, H.,*** *et al.,* Heterodimerization of Msx and Dlx homeoproteins results in functional antagonism. *Mol Cell Biol, 1997.* ***17****(5): p. 2920-32.*

341. ***Zhu, L.,*** *D. Lovinger, and E. Delpire,* Cortical neurons lacking KCC2 expression show impaired regulation of intracellular chloride. *J Neurophysiol, 2005.* ***93****(3): p. 1557-68.*