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

Ph.D. Dissertation

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1.INTRODUCTION

γ-Aminobutyric acid (GABA) is the major inhibitory neurotransmitter in the brain and as such plays a key role in the control of neuronal excitability. The heterogeneity in types of GABAergic synapses and interneurons unraveled in the last decade indicate that an array of GABAergic signaling functions might exist in the brain. GABA is involved in a variety of biological functions such as locomotor activity, learning, reproduction, circadian rhythms as well as cognition, emotions and sleep. Furthermore, GABA has been implicated in modulating of nearly all key cellular events including proliferation, migration, axonal growth, differentiation, synapse formation and cell death in the developing CNS.

Beside its function in the CNS, GABA has been also detected in many peripheral neuronal and non-neuronal tissues and suggested to be involved in physiology of glia cells, digestive tract, pancreas, liver, adrenal medula, respiratory and kidney epithelium, osteoblasts, chondrocytes, and germ cells.

The pleiotropic effects of GABA signalling may be achieved through the diversity of components responsible for its synthesis, release, signal propagation, reuptake and degradation. In the mammalian brain, **GABA** is synthesized primarily from glutamate in a reaction that is catalysed by two glutamic acid decarboxylase (**GAD**) enzymes, GAD65 and GAD67, which are co-expressed at variable ratios in the GABAergic neurons. The two GAD isoforms are highly homologous at the protein level, but show different affinity to the cofactor pyridoxal phosphate (PLP) and distinct subcellular localization: GAD65 is predominantly localized to nerve endings and is thought to synthesize GABA for vesicular release, whereas GAD67 is enriched in the cytoplasm and is probably involved in the synthesis of the cytoplasmic GABA pool. During mouse and rat embryonic development two additional, alternatively-spliced forms of GAD67 gene (I80 and I86) are synthesized coding for the truncated **GAD25** and enzymatically active embryonic **GAD44** that are transiently expressed in a strict temporal order during neuronal differentiation and latter thought to synthesize a GABA pool used for paracrine signalling. GABA is loaded by the vesicular GABA transporter (**VGAT**) into synaptic vesicles and is released from nerve terminals by calcium-dependent exocytosis. However, nonvesicular forms of GABA secretion have also been described that might be particularly important during development. GABA exerts its effect through activation of ionotropic $\mathbf{GABA_A}$ and $\mathbf{GABA_C}$ or metabotropic $\mathbf{GABA_B}$ receptors. $GABA_A$ receptor-chloride channels are pentameric assemblies of distinct subunits composed of 2α (α1-6), 2β (β1-3) and 1γ (γ1-3) or δ,π,θ) subunits, respectively. $GABA_AR$ display unique regional and temporal expression profiles during brain development and exhibit distinct pharmacological and electrophysiological properties dependent on the subunit composition. The

functional metabotropic $GABA_B$ receptor is an obligatory heterodimer composed of $GABA_{BR1}$ and $GABA_{BR2}$ receptor subunits. $GABA_BR$ mediates delayed postsynaptic inhibition and presynaptic neurotransmitter release. $GABA_BR$ activation can also induce changes in gene expression by activating second messenger pathways through coupling to G-proteins . GABA signals are terminated by reuptake of the neurotransmitter into nerve terminals and/or into surrounding glial cells or the presynaptic GABAergic neuron by a class of plasma-membrane GABA transporters (**GATs**)

The major objective of this thesis was to characterize the GABA signalling components and functionality during differentiation using two different model systems: mouse embryonic stem (ES) cells and the ocular lens.

Embryonic stem (ES) cells are pluripotent cell lines derived from the inner cell mass (ICM) of the blastocyst. In the presence of Leukemia inhibitory factor (LIF) ES cells undergo symmetric divisions and remain in undifferentiated state while maintaining the potential to contribute to all tissues found in the adult body with the exception of the placenta. These cells provide a valuable model to study signalling systems (pathways) in the undifferentiated state and during embryonic differentiation *in vitro*.

The vertebrate lens is composed of two populations of cells: a layer of cuboidal epithelial cells at the anterior surface, and elongated fibre cells at different stages of differentiation that constitute the bulk of the tissue. In a sequence of events precisely regulated by multiple transcription and growth factors single epithelial cells of the germinative zones proliferate and move to the equator of the lens. In the transitional zone found below the equator, the epithelial cells elongate and differentiate into fibre cells that move in to fill up the lumen of the lens. Parallel with maturation, fibre lens cell loose all membranous organelles, increase their volume and expression of the lens crystallins, which provide for the lens transparency required for proper vision. This regional compartmentalization makes the lens an attractive model system to study signalling mechanisms during cellular differentiation.

2. METHODS USED IN THIS STUDY

»» ANIMAL HANDLING

»» CELL CULTURE

- »»Preparing mouse embryo fibroblasts (MEF)
- »»ES cell culture
- »» ES cell neuronal differentiation procedure
- »» Primary lens epithelial culture

»» GENE EXPRESSION ANALYSIS

- »» RNA isolation from cells and tissues
- »» First-strand cDNA synthesis
- »» PCR amplification
- »» Semi-quantitative RT-PCR analysis

»» WESTERN BLOT ANALYSIS

- »» For cell culture
- »» For mouse lens

»» GABA MEASUREMENT BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

»» ELECTRON MICROSCOPY

»» IMMUNOHISTOCHEMISTRY

»» IMMUNOCYTOCHEMISTRY

»» $[CA^{2+}]_i$ IMAGING STUDIES

- »» Measurements of change of intracellular Ca^{2+} concentration in the undifferentiated ES cells
- »» Measurements of change of $[Ca^{2+}]_i$ in the lens cells
  - »» Intact lens
  - »» Primary LEC culture

»» CELL PROLIFERATION STUDIES

3. OBJECTIVES/AIMS

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.

4. RESULTS AND DISCUSSION

1. UNDIFFERENTIATED MOUSE ES CELLS EXPRESS FUNCTIONAL GABA SIGNALLING

All structural components of GABA signalling 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 derived from and developmentally closely related to the ICM of the mouse blastocyst express structural components needed for assembly of functional GABA signalling: GABA, GABA synthesizing enzymes (GAD65 and EGADs), membrane and vesicular GABA transporters (GAT1 and VGAT), as well as multiple $GABA_A$ ($\alpha1,\alpha3,\alpha4,\alpha5,\beta3,\gamma2S,\gamma3,\delta,\varepsilon$) 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 GABAR are engaged in intensive cell-cell communications within the ES colony.

Activation of $GABA_A$ and $GABA_B$ receptors leads to increase of intracellular Ca^{2+} level in ES cells

The presence of multiple subunits in mouse ES cells indicates that the assembly of functional GABA receptors of both ionotropic and metabotropic type is possible. We tested this assumption by monitoring the changes in $[Ca^{2+}]_I$ in ES cells upon bath application of GABA (1mM) and muscimol (30 μM) or baclofen (20 μM), selective agonists for $GABA_AR$ and $GABA_BR$, respectively. Both agonists induced a sharp increase of fluorescence intensity in ES cells, but not in fibroblasts. The average number of cells responding to GABA, muscimol or baclofen and the amplitude of their responses differed slightly. While the $GABA_AR$ response was completely blocked by the specific $GABA_AR$ antagonist bicuculline, the Ca^{2+} transients induced by baclofen could not be blocked by the potent $GABA_BR$ antagonists CGP 55845 or CGP 54626, which indicates that $GABA_BR$ of ES cells possess unique pharmacological properties.

Furthermore, we found that in the presence of CPA the number of muscimol-evoked Ca^{2+}transients was reduced, but their amplitude remained unchanged, while baclofen-induced $[Ca^{2+}]_i$ increase was nearly completely blocked by CPA. The voltage–gated calcium channel (VGCC) blocker cadmium (100 μM) significantly reduced the amplitude of Ca^{2+} transients but not the number of cells displaying calcium transients

induced by muscimol or baclofen. In addition, baclofen-evoked Ca^{2+} responses were largely independent of extracellular Ca^{2+}.

Therefore, our data suggest that $GABA_AR$-evoked $[Ca^{2+}]_i$ rise in ES cells has a dual origin: influx from the extracellular space and release from intracellular stores, whereas $GABA_BR$-mediated Ca^{2+} transients originate exclusively from internal Ca^{2+} stores. With respect to $GABA_AR$, ES cells share similarities with immature neurons where $GABA_AR$-mediated elevation of $[Ca^{2+}]_i$ occurs mainly through VGCC-mediated Ca^2 influx, whereas $GABA_BR$-mediated Ca^{2+}response shows unique features that show partial similarities with $GABA_BR$ of primary astroglial cultures.

GABA receptors as modulators of cell proliferation in ES cells

The presence of GABA signalling suggested that it could play a role in the cell proliferation and/or differentiation of ES cells. We tested this assumption by studying the effect of chronic GABA and GABAR agonists treatment on the cell numbers of exponentially growing cultures, 24/48 hrs following addition of the drugs. No statistically significant differences in the cell numbers were encountered after treatment with the GABA or muscimol, while baclofen treatment increased the total cell numbers and clone sizes after 48h incubation time. Similarly, incubation with the highly specific $GABA_AR$ antagonist SR95531 produced a similar effect as baclofen by nearly doubling the cell number (average clone size) compared to control.

In conclusion, our results suggest that activation of both $GABA_A$ and $GABA_B$ receptors induces rise in the intracellular calcium concentration, but affects diversely the proliferation of undifferentiating mouse ES cells.

2. GABA SIGNALLING DURING IN VITRO NEURONAL DIFFERENTIATION OF MOUSE ES CELLS

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 COMPONENTS AND FUNCTIONALITY OF THE GABA SIGNALING DURING MOUSE LENS DEVELOPMENT

Sequentially induction of different GAD forms recapitulates specific stages of lens fiber differentiation: correlation with the transcriptional factors Dlx2 and Dlx5

The spatio-temporal regulation of GABA and GAD in the mouse lens was studied from early developmental stages into the adult (E14.5-P30). All four GAD forms are expressed during lens development-GAD65 and three protein forms encoded by GAD67 gene (GAD25, GAD44 and "adult" GAD67). During earlier embryonic stages (E14.5-E16.5) predominantly GAD65 and the embryonic forms GAD25 and GAD44 were expressed, while adult GAD67 was extremely low. In the E9.5-E16.5 mouse lens, the GAD65 and EGAD localized preferentially at the apical and basal tips of elongating primary fibres and gradually disappear concomitant with fibre maturation and nuclear degradation, which indicates that the early GADs may play a role in **primary fibres'** elongation and final steps of differentiation characterised by induction of crystalline synthesis and degradation of all membrane organelles. GAD67 is up-regulated after E17.5 in the elongating equatorial secondary fibres, an indication that GAD67 is involved mostly in the differentiation of **secondary fibres**, a process that continues throughout life. Thus the lens GAD expression is characterized with a unprecedented segregation of the two GAD forms. The GABA content of the

lens at different developmental stages showed a clear correlation with the temporal profile of GADs.

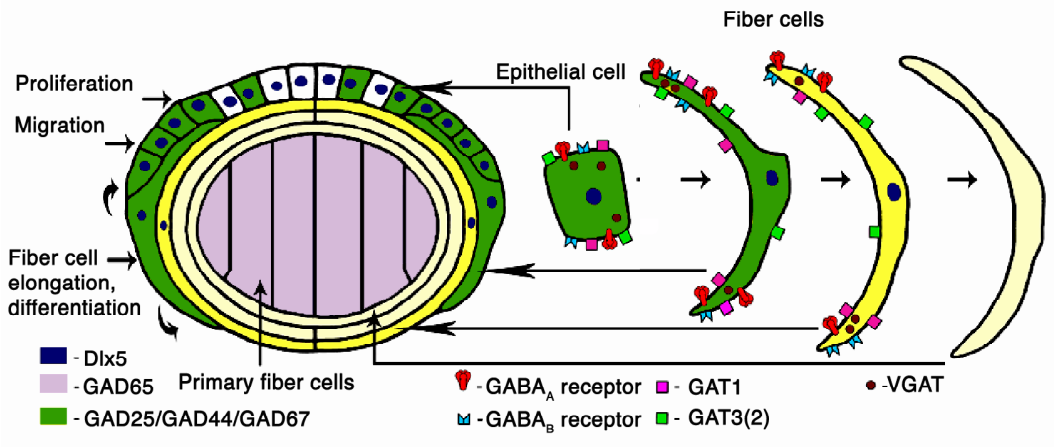

Fig.1. Expression patterns of different GABA signaling components in the neonatal mouse lens: a schematic presentation.

We also examined the presence of Dlx2 and Dlx5- transcription factors expressed in spatially restricted domains of the developing forebrain that overlap with the expression domains of GAD. Dlx2 and Dlx5 have been shown to induce GAD expression in embryonic brain and are thought to be transcriptional regulators of both GAD genes. Our semi quantitative RT-PCR showed that similar to the developing forebrain, Dlx2 precedes Dlx5 expression: Dlx2 mRNA temporal expression is similar to that of the early GAD forms (EGAD and GAD65), while Dlx5 mRNA, correlates, albeit only partially with that of GAD67. These findings strongly suggest that Dlx5 may be needed for induction, but not maintenance of expression of GAD67. Further experiments will be needed to verify this hypothesis.

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

In addition to different GAD forms, GABA$_A$R subunits ($\alpha_{1,2,3\ 4}$; $\beta_{1,\ 2,\ 3}$; $\gamma_{1,\ 2,\ 3}$ and δ), both GABA$_B$R subunits, the vesicular and membrane GABA transporters show dynamic spatio-temporal profiles during developmental stages E14.5-P30 that could be grouped according to developmental stage and subunit type. Our RT-PCR data indicate a clear switch from embryonic to postnatal expression of different components. Thus, the subunit composition of GABA$_A$R 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. Notable is the exceptionally high expression of γ_3 and β_3 and the total absence of the closely positioned α_5 indicate that they are oppositely regulated at the transcriptional level throughout lens development. We also detected the expression of both GABA$_B$ receptor subunits in the developing

lens. However, the extremely low level of $GABA_BR2$ in the postnatal lens may suggest that $GABA_BR1$ assembles with other proteins.

Based on Spearmann correlation analysis of the expression of the molecular components of GABA signaling, we suggest, that in the embryonic lens GABA synthesized by GAD65 is released predominantly by VGAT and binds to $GABA_AR$ $\alpha_{2,3}$ subunit-containing receptors, while 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 and is the embryonic counterpart of GAD67 may be released by inversion of one or more membrane GATs, as previously suggested.

Functional GABA receptors in the developing lens: application of GABA triggers rise of intracellular calcium in the neonatal lens

To test the functionality of the GABA signaling in intact lenses we performed Ca^{2+} imaging using LSM and a specially designed perfusion system. GABA evoked an increase in fluorescence intensity mostly in cells of the equatorial region that are undergoing transition from epithelial to fiber cells. A drawback of this method was that only a small fraction of $GABA^+$ cells in the P0 lens could be labeled with Ca^{2+} sensing dyes and borders of the reacting cells were poorly difined. To characterize the specific 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. Lens cells in our cultures represented different differentiation stages (epithelial cells, nucleated and anucleated fibers) found in native lenses and are in addition they were far more permeable for Ca^{2+} imaging dyes. 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 signalling: GAD, GABA transporters and GABA receptors.

Both muscimol ($GABA_AR$ agonist) and baclofen ($GABA_BR$ agonist) evoked Ca^{2+} transients, predominantly in lentoids and sheets of epithelial cells that could be blocked by the selective antagonists bicuculline and CGP55845, respectively.

Wave-propagation of the signal and persistent synchronous oscillations were often observed upon application of GABA or baclofen, but not muscimol, which suggests the involvement of gap junctions previously shown to mediate Ca^{2+} waves in primary LEC upon mechanical stimulation. Persistent Ca^{2+} elevation causes gap junction uncoupling and formation of hemichannels, implying that $[Ca^{2+}]_i$. rise induced by GABAR activation may regulate gap junction formation, which is vital for lens ion homeostasis. Gap junction-mediated adhesion has also been implicated in the

migration of neuronal progenitors in the cortex.

5. MAIN FINDINGS:

1. All molecular components of GABA signalling: GABA, GADs, $GABA_A$ and $GABA_B$ receptor subunits and GABA transporters are expressed in the undifferentiated mouse ES cells providing a structural basis for synthesis, release, receptor-binding and re-uptake of GABA.

2. $GABA_A$ and $GABA_B$ receptor proteins are transported to the ES cell membrane and could form functional receptors. Activation of $GABA_A$ and $GABA_B$ receptors induce a transient increase of intracellular Ca^{2+}confirming that embryonic stem cells possess functional GABA receptors. In addition, that $GABA_AR$- evoked $[Ca^{2+}]_i$ rise in ES cells has a dual origin: Ca^{2+} influx and intracellular store release, whereas $GABA_BR$-mediated Ca^{2+} transients originate exclusively from internal stores.

3. GABA, acting as an auto/paracrine factor can module proliferation rate in the ES cells: acting on $GABA_BR$ it stimulates, whereas $GABA_AR$ activation inhibits cell proliferation.

4. Molecular components of GABA signalling and GABA are regulated in a developmental stage-specific manner staring from very early steps of neuronal differentiation.

5. GABA and all forms of its biosynthetic enzyme GAD are expressed in the mouse lens from earliest developmental stages. Our results demonstrate that the GAD forms display developmental stage-specific expression: GAD65 and EGAD predominating during primary fiber differentiation, and GAD67 being most abundant in the postnatal secondary fiber cells.

5. GABA can be detected from the earliest stage of the lens development and peaks during the most extensive secondary fiber cell differentiation and elongation.

We also demostrated that Dlx2 and Dlx5 transcription factors are expressed sequentially in the developing lens. Dlx2 expression is induced before Dlx5. The temporal expression of Dlx2 parallels that of EGAD and GAD65, suggesting that Dlx2 may be needed for induction and/or maintenance of the “early” GAD forms.

Dlx5 expression correlates only partially with that of GAD67: its initial up-regulation precedes that of GAD67, but unlike GAD67, Dlx5 is not expressed beyond P14 suggesting that Dlx5 may be needed for induction, but not for maintenance of GAD67 expression.

Different $GABA_A$ and $GABA_B$ receptor subunits, GABA transporters (vesicular VGAT and membrane GAT) are expressed in the developing mouse lens as well as in primary epithelial lens cultures

Both $GABA_A$ and $GABA_B$ receptor activation evoked transient increase of $[Ca^{2+}]_i$ in intact lenses and in primary LEC cultures, demonstrating the presence of functional GABA signaling in the developing lens.

6. 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 expression segregation of GABA signalling components along the two GAD proteins, the lens has proven a unique model system affording the possibility to study the subcellular roles of GAD65 and GAD67 and components downstream of each gene, especially in view of their correlated regulation during development and in the adult.

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