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Quantitative characterization of regional differences in the $GABA_A$-receptor α1-subunit mRNA expression in the rat brain

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Abstract

Inhibition in the central nervous system is largely mediated by local-circuit neurons that release GABA (γ-amino-butyric acid). $GABA_A$-receptors play a major role in virtually all brain physiological functions and serve as targets for numerous classes of drugs, used both in clinical practice and as research tools. These receptors are heteropentamers, α1 being the most widely occurring subunit; therefore it is the best candidate to be studied in pathological conditions where the inhibitory system might be altered (e.g. epilepsy). We compared quantitatively the regional distribution of $GABA_A$-receptor α1-subunit ($GABA_AR$–α1) expression in three brain areas: neocortex, hippocampus and cerebellum by RT–qPCR. TaqMan probe was used in order to avoid detection of non-specific amplification products and synaptophysin as internal control. This substance was chosen because it has a stable expression restricted to neurons, and contrary to GAPDH, the most commonly used reference gene for expression analysis, synaptophysin expression is not modified in animal models of epilepsy. Expression of synaptophysin was higher than expression of $GABA_AR$-α1 in all samples from the central nervous system. The latter was significantly different among the studied brain areas. It was the smallest in the hippocampus, intermediate in the neocortex and the highest in the cerebellum. Interanimal differences were small for any brain region under study. These results indicate that combination of TaqMan real-time PCR method with synaptophysin as internal control can reliably measure the relative expression of $GABA_AR$–α1 mRNA, and are suitable for investigating the modifications that appear under pathological conditions and/or diverse experimental paradigms.

Keywords: $GABA_A$-receptor, α1-subunit, real-time PCR, TaqMan, synaptophysin, epilepsy.

Introduction

The excitatory neuronal networks of the central nervous system are controlled by inhibitory innervation that governs the threshold of activation, pattern of action potential firing and modification of synaptic strength. This inhibition is largely mediated by local-circuit neurons that release GABA (γ-amino-butyric acid) [1]. GABA produces its inhibitory effect by interacting with two classes of receptor molecules on the target cells: $GABA_A$-receptors, which are ligand-gated anion channels and $GABA_B$-receptors, which are coupled to G-protein-mediated cellular responses. Because of their widespread localization throughout the mammalian nervous system, $GABA_A$-receptors play a major role in virtually all brain physiological functions and serve as targets of numerous classes of drugs, used both clinically and important as research tools. These receptors are assembled from a family of 19 homologous subunit gene products (α1–6, β1–3, γ1–3, δ, ε, θ, π and ρ1–3) and form numerous, mostly hetero-oligomeric, pentamers [2]. The expression of these subunits is highly heterogeneous, each displaying a unique regional distribution pattern. Alpha-1 is the most widely occurring subunit and is considered to be ubiquitously present in the brain [3]; therefore it is the best candidate to be studied in pathological conditions where the inhibitory system might be altered (e.g. epilepsy).

Analysis of subunit expression is important to understand the pharmacology and functional significance of $GABA_A$-receptor subunits in various brain regions. There is no pathological state with complete loss of inhibition, but quantitative modifications appear. For this reason, the traditional qualitative assessment of the receptor subunit distribution must be complemented with a quantitative measurement of the expression levels. The normal distribution of the $GABA_A$-receptor subunit mRNAs in rat was determined by *in situ* hybridization [3–5]. Reverse transcription followed by PCR (Polymerase Chain Reaction) is a standard technique used for detecting gene expression, but its sensitivity is very limited.

More recently, real-time PCR has been used for more sensitive and reliable quantification of $GABA_A$-receptor subunit expression in brain tissue samples. The "TaqMan" real-time PCR measures PCR-product accumulation during the exponential phase of the PCR reaction using a dual-labeled fluorogenic probe. The TaqMan probe avoids detection of non-specific amplification products because of its stringent design to bind to the target gene sequence, therefore this method

is superior to the intercalating SYBR green that binds to all double-stranded DNA-products.

Recently, a systematic optimization of TaqMan real-time PCR protocol for $GABA_A$-receptor subunit superfamily was published [6]. This study used RNA extracted from whole brain, so to obtain information regarding the regional distribution of the $GABA_A$-receptor subtypes further investigation is needed. Real-time PCR requires that a non-regulated reference gene is used as endogenous control for relative analysis of gene expression. For this purpose, the study of Gangisetty O and Reddy DS [6] used glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

Ideally, internal control transcripts should show constant, high level expression independent of experimental conditions. However, using pilocarpine-induced epilepsy, both real-time RT–PCR and *in situ* hybridization revealed a striking up-regulation for GAPDH, the most commonly used reference gene for expression analysis [7]. These changes in expression can introduce a large error into mRNA quantification based on normalization to GAPDH. When GAPDH, β-actin, cyclophilin A, hypoxanthine phosphoribosyltransferase (HPRT) and synaptophysin was systematiccally tested by comparing pilocarpine-treated and control animals, the most stable expression was observed for synaptophysin [7]. The use of synaptophysin as endogenous control has the additional advantage that its expression is restricted to neurons, so mRNA levels will not be affected by an increase in the non-neuronal cells also present in brain samples.

In this study, we compare quantitatively the regional distribution of $GABA_A$-receptor α1-subunit expression in three brain areas: neocortex, hippocampus and cerebellum, by combining the advantages of TaqMan real-time PCR method and the remarkably stable expression of synaptophysin.

Material and Methods

The tissue was obtained from five male Wistar rats (200–300 g) with free access to food and water. Environmental conditions for housing of the rats, and all procedures that were performed on them, were in accordance with the European Communities Council Directive (86/609/EEC) and the Ethical Guidelines of the University of Medicine and Pharmacy of Targu Mures. The animals were deeply anesthetized with Ketamine/Xylazine (90, respectively 10 mg/kgbw, i.p.) and then transcardially perfused with saline (0.9% NaCl, 4^0C, for 1.5 minutes). The whole brain was removed and sectioned in ice-cold saline. One or two coronal slices (approx. 1 mm thickness) were cut at the middle rostro-caudal extent of the brain; the entire hippocampus was removed from the slice with the best visualization of the hippocampal region. From the same slice, two blocks of neocortex was collected (dorsal region, approx. 3–4 mm^3). One block of tissue with the same size was collected from a sagittal section through the cerebellar vermis. As non-neural tissue, a block from the deflated lung was removed.

The tissue blocks were immediately processed. Five to eight samples were collected from one animal, and these were processed sequentially, therefore to prevent RNA decomposition, RNA later solution was used (Ambion Inc, USA) in 10:1 proportion. Approximately 30 mg tissue was homogenized in 600 μL RLT-lysis buffer (Qiamp Kit component) using a Potter–Elvehjem Tissue Grinder (Wheaton Science). Total RNA was extracted with Qiamp RNA Blood Mini Kit (Qiagen Group), according to the manufacturer's instructions. The RNA was diluted in 40 μL ultra pure water, and the RNA concentration was determined by NanoDrop ND–100 Spectrophotometer (Thermo Scientific, USA).

Total RNA from each sample was used to synthesize cDNA using High Capacity cDNA Reverse Transcription Kit according to the manufacturer's instructions (Applied Biosystems, USA) with Gene Amp PCR System 9700 (Applied Biosystems, USA). A final concentration of 1 μg/100 μL was obtained.

TaqMan real-time quantitative PCR amplification reactions were carried out in an AB 7500 Real–Time PCR System (Applied Biosystems) using TaqMan Universal PCR Master Mix (Applied Biosystems) – 40 ng cDNA and 10 μL 2 × TaqMan Buffer with standard conditions (initial setup 2 minutes – 50^0C, 10 minutes – 95^0C, denaturation 15 seconds – 95^0C, annealing 1 minutes – 60^0C) and 50 cycles.

Synaptophysin was used as reference gene with the following forward (f) and reverse (r) primers: (f), 5'-TCAGGACTCAACACCTCAGTGG-3'; (r), 5'-AA CACGAACCATAAGTTGCCAA-3'; and the hybridization probe: F5'-TTTGGCTTCCTGAACCTGGTGCT CTG-3'T (Applied Biosystems). All reactions were performed in a 20 μL volume with 6 pmol forward and reverse primer and 4 pmol TaqMan probe.

$GABA_A$-receptor α1-subunit expression was studied using GABRA1-gene TaqMan primer probe (Rn00788315_m1, Applied Biosystems) according to the manufacturer's instructions.

All reactions were executed in triplicate. In the case of negative control, cDNA was not added.

The relative quantification of $GABA_A$-receptor α1-subunit expression was performed by calculating the concentration difference (Y) against the reference gene:

$$Y = 2^{-\Delta Ct}$$

where

$\Delta Ct = Ct(GABA_A\text{-}receptor\ \alpha 1) - Ct(synaptophysin)$;

Ct – threshold cycle;

3.32 cycle difference meaning 10-fold concentration difference.

For statistical analysis, GraphPad Prism was used. (HE).

Results

The $GABA_A$-receptor α1-subunit expression was analyzed in neural and extraneural tissue from five rats. The exsanguination and perfusion with ice-cold saline resulted in adequate solidification of the brain, which could be removed unscathed from the scull. On average 1 minute elapsed from the opening of the thoracic cavity until the insertion of transcardial perfusion and further 4–5 minutes until the brain was removed from the skull.

The brain of the first animal was processed on a humidified filter paper, at room temperature. To prevent RNA deterioration, tissue blocks were cut as quickly as possible (less than two minutes) with a scalpel blade under naked eye examination. This method, although very rapid, proved to give poor precision regarding the delimitation of the region of interest. Another problem was that at room temperature the brain tissue started to melt and stuck to the filter paper. To alleviate these problems, further experiments were carried out using a dissecting microscope and the blocks were cut in 4^0C liquid, in a Petri dish prepared in advance with frozen saline. This method permitted to section out regions with similar amount of white matter, from similar brain areas in all animals. The collection of all neural tissue blocks with this method took approximately 5 minutes. Results are based only on the last four animals.

In all investigated brain areas, the expression of synaptophysin was higher than the expression of the $GABA_A$-receptor α1-subunit (Figure 1, a–c). The smallest relative expression level was found in the hippocampus, the largest in the cerebellum (Table 1). The neocortex had intermediate expression levels. The mean expression level was significantly different for any combination of the regions (hippocampus *vs.* cortex, hippocampus *vs.* cerebellum, cortex *vs.* cerebellum, Student *t*-test, $p<0.01$). In the lung, the situation is completely different: here the expression level of the $GABA_A$-receptor α1-subunit was ten-fold higher than the expression of synaptophysin (Figure 1d).

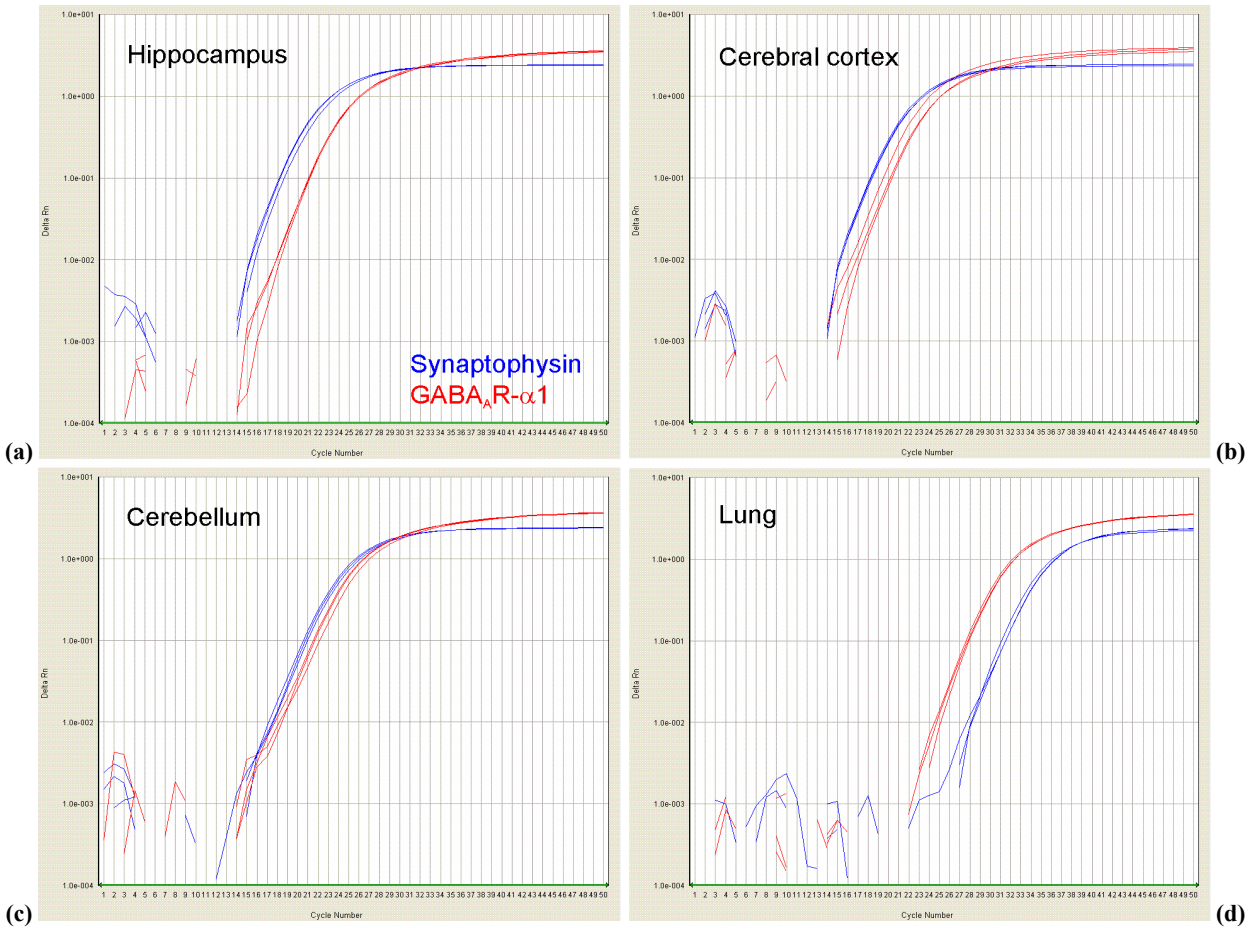
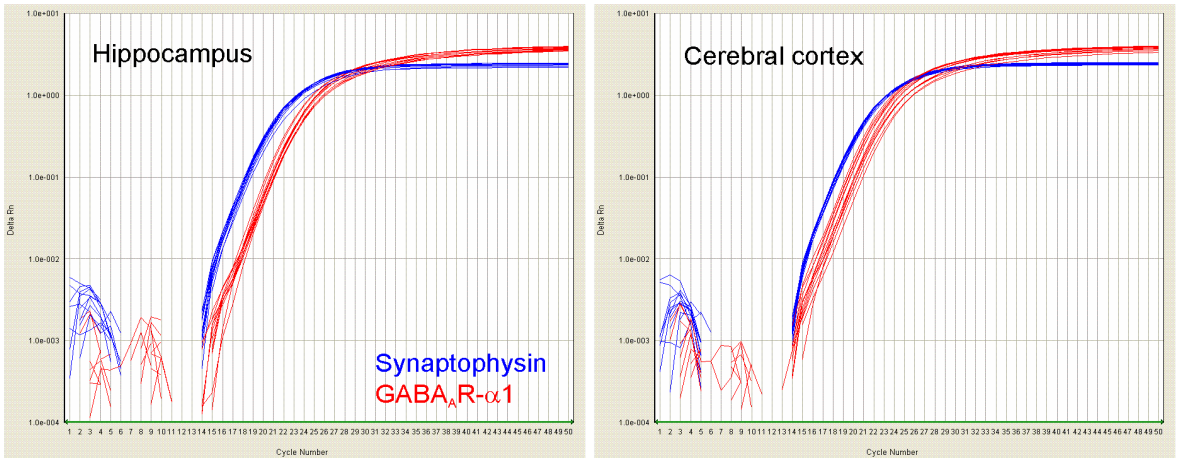


Figure 1 – ***(a–d) Results of RT–PCR reactions for the investigated brain regions and the lung. Traces represent the amplification curve obtained for samples from one animal. All reactions were performed in triplicate.***

Table 1 – ***$GABA_A$-receptor α1-subunit mRNA concentration in proportion (%) to the endogenous control, synaptophysin (mean ± standard error)***

Cerebral cortex	29.8 ± 2.1
Hippocampus	18.7 ± 2.5
Cerebellum	72.4 ± 8.6
Lung	1033.8 ± 145.5

The inter-animal differences were small for any region under study. Overplotting traces of the exponential amplification phase for all animals, an almost perfect overlap was found for synaptophysin (Figure 2, a and b). This result suggests that synaptophysin has a very constant expression pattern in the brain. In case of $GABA_A$-receptor α1-subunit the data was more scattered, but interregional differences were higher than inter-animal differences (Figure 3). Two-way ANOVA revealed that interregional difference accounts for 86.5% of the total variance of the sample in contrast with the 5.7% inter-animal difference (7.8% residual variance not related to systematic differences). Variation among the three brain areas was significantly greater than expected by chance ($p<0.001$, Kruskal–Wallis non-parametric ANOVA-test), whereas variation among animals was not significantly greater ($p=0.90$).



(a) (b)

Figure 2 – ***(a, b) Cumulative results of RT–PCR reactions for four animals. Observe the almost perfect superposition of the amplification phase for synaptophysin.***

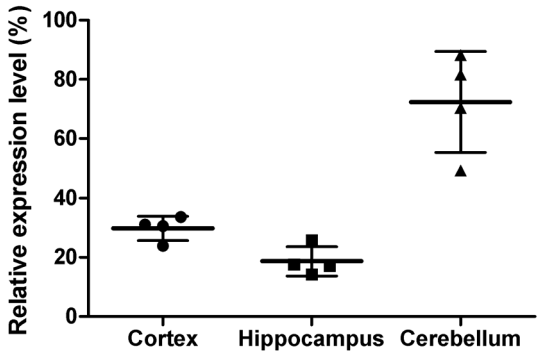


Figure 3 – ***Regional distribution of GABA$_A$R-α1 mRNA in different brain areas. Each symbol shows the result for one animal. The bars represent mean (thick line) ± standard deviation (thin line) for the given region.***

Discussion

In an *in situ* hybridization study regarding the distribution of 13 GABA$_A$-receptor subunit mRNAs in the rat brain [3] it was found that α1-subunit is present in almost every brain region, but the signals obtained with 33S-labeled oligonucleotide probes was dissimilar in different regions. The results of this semiquantitative study (Table 2) are in line with our results.

Table 2 – ***Distribution of GABA$_A$-receptor α1-subunit mRNA according to Wisden W et al. [3]***

Region	Signal strength
Neocortex	
layer II/III	++
layer IV	+
layer V/VI	++
Pyriform cortex	+++
Hippocampus	
CA1 str. pyramidalis	++
CA3 str. pyramidalis	+
dentate gyrus granule cells	++
Tenia tecta	+
Cerebellum	
Purkinje cells	+++
Stellate/Basket cells	+++
Granule cells	+++

+++: intense; ++: strongly positive; +: positive.

The significantly higher expression level found in the cerebellum is in accord with Wisden's study, where all major cell types were intensely labeled for the α1-subunit mRNA. Our results show also significantly higher expression level in the neocortex than in the hippocampus. In Wisden's study, the neocortex and hippocampus appeared similarly labeled, but the strong signal in the pyriform cortex and weak signal in the tenia tecta (which has similar embryonic origin with the hippocampus) suggests that there are small differences between these regions, which are detectable only at quantitative level.

It is not certain that mRNA levels reflect accurately the receptor protein level in the cell membrane, which may be affected by regulatory mechanisms of translation and intracellular trafficking. Furthermore, the protein might be located in dendrites or axon terminals far from the soma where the mRNA resides. Immunohistochemical studies have indicated a similar distribution pattern for the α1-subunit protein (Table 3) [8, 9].

In our study tissue blocks containing all layers were collected, therefore it can be expected to reflect the average expression level of the α1-subunit in these layers jointly. A rough average of the immunohistochemical data (Table 3) [8] indicates highest α1-subunit protein concentration in the cerebellum, lowest in the hippocampus and intermediate in the neocortex, what is in consonance with our results.

Table 3 – ***Regional distribution of GABA$_A$-receptor α1-subunit immunoreactivity [8]***

Region	Signal strength
Cerebral cortex	
all layers	++
outer layers	++
inner layers	++
Hippocampus	
molecular layer	+
hilar neurons	++
str. oriens/radiatum	++
Cerebellum	
granule cell layer	+++
molecular layer	++

+++: extremely high; ++: high; +: low.

At pharmacological and behavioral level, GABA$_A$-receptors are modulated by benzodiazepines. Zolpidem shows preferential binding to GABA$_A$-receptors con-

taining α1-subunits. Zolpidem administration in non-human primates indicates that α1-subunit containing receptors are critically involved in sedative and motor effects of benzodiazepines, but they do not play a key role in the anxiolytic effect [10]. Our results support this observation, as high expression of the α1-subunit in the cortex and cerebellum might explain the sedative and motor effects, while the low expression in the hippocampus, a limbic structure, could explain the lesser anxiolytic effect.

In the lung, expression of functional ionotropic GABA-receptors was shown recently on the apical plasma membrane of alveolar cells [11]. These receptors contribute to alveolar fluid homeostasis via luminal secretion of Cl^-. The source of GABA seems to be the alveolar type II cells, because GABA and its synthesizing enzyme, glutamic acid decarboxylase (GAD), were expressed in these cells [11]. It is supposed that type II cell-originated GABA may regulate Cl^- transport in type II cells through an autocrine pathway and in type I cells via a paracrine mechanism. The local, non-neuronal origin of GABA can explain our result, the ten-fold higher expression of $GABA_A$-receptor α1-subunit compared with the expression of synaptophysin (a neuron specific protein).

Conclusions

$GABA_A$-receptor α_1-subunit mRNA could be reliably measured by RT–qPCR in all investigated brain areas. The TaqMan real-time PCR method could be successfully combined with the use of synaptophysin as internal reference. In all our measurements synaptophysin had very similar expression in any given brain area, all traces of the exponential amplification phase could be almost perfectly superimposed.

In all samples from the central nervous system, the expression of synaptophysin was higher then the expression of the $GABA_A$-receptor α1-subunit. In the lung an inverse relation was found, the α1-subunit expression was ten-fold higher than the expression of synaptophysin. The $GABA_A$-receptor α1-subunit expression was significantly different among the studied brain areas. It was the smallest in the hippocampus, intermediate in the neocortex and the highest in the cerebellum.

The interregional differences were significant, but on the other hand, inter-animal differences were small for any brain region under study. These results indicate that the method utilized by us is suitable to measure $GABA_A$-receptor α1-subunit mRNA alteration under pathological conditions and/or diverse experimental paradigms.

Acknowledgements

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