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The Optimization of TaqMan Real-Time RT-PCR Assay for Transcriptional Profiling of GABA-A Receptor Subunit Plasticity

Omkaram Gangisetty and Doodipala Samba Reddy

Department of Neuroscience and Experimental Therapeutics, College of Medicine, Texas A&M System Health Science Center, College Station, Texas 77843-1114, USA

Abstract

The GABA-A receptor plays a critical role in inhibitory neurotransmission in the brain. Quantitation of GABA-A receptor subunits in various brain regions is essential to understand their role in plasticity and brain disorders. However, conventional RNA assays are tedious and less sensitive for use in studies of subunit plasticity. Here we describe optimization of a sensitive assay of GABA-A receptor subunit gene expression by TaqMan real-time PCR. For each subunit gene, a set of primers and TaqMan fluorogenic probe were designed to specifically amplify the target template. The TaqMan methodology was optimized for quantification of mouse GABA-A receptor subunits (α_{1-6} , β_{1-3} , γ_2 , and δ) and GAPDH. The TaqMan reaction detected very low levels of gene expression (~100 template copies of cDNA). A standard curve for GAPDH and one of the target genes, constructed using the cDNA, revealed slopes around -3.4 ($r^2=0.990$), reflecting similar optimum PCR efficiencies. The methodology was utilized for quantification of the GABA-A receptor α_4 subunit, which is known to upregulate following withdrawal from chronic progesterone or neurosteroids. Our results show that the α_4 -subunit expression increased threefold in the hippocampus following neurosteroid withdrawal in mice. The TaqMan PCR assay allows sensitive, high-throughput transcriptional profiling of complete GABA-A receptor subunit family, and thus provides specific tool for studies of GABA-A receptor subunit plasticity in neurological and psychiatric animal models.

Keywords

Real-time PCR; TaqMan; GABA_A receptor; Neurosteroid withdrawal; GAPDH

1. Introduction

GABA (γ -aminobutyric acid) is the major inhibitory neurotransmitter in the brain where it acts at GABA-A and GABA-B receptors. The GABA-A receptor is a transmembrane-gated ion channel that mediates both phasic inhibitory synaptic transmission and tonic perisynaptic inhibition, and thereby plays a critical role in the pathophysiology of neuropsychiatric conditions including anxiety and epilepsy. The GABA-A receptor has binding sites for GABA, benzodiazepines and neurosteroids (Hosie et al., 2007), and therefore, it is a major target receptor for several clinically used anxiolytic, antiepileptic and anesthetic agents. The receptor

Address correspondence to: D. Samba Reddy, Ph.D., R.Ph., Associate Professor, Department of Neuroscience and Experimental Therapeutics, College of Medicine, Texas A&M Health Science Center, 228 Reynolds Medical Building, College Station, Texas 77843-1114, USA, Tel: 979-862-2852; Fax: 979-845-0790, E-mail: E-mail: reddey@medicine.tamhsc.edu.

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exists as a pentamer of 4 transmembrane subunits that form an intrinsic chloride channel. Seven different classes of subunits with multiple variants have been reported in mammals (α_{1-6} , β_{1-3} , γ_{1-3} , ρ_{1-3} , δ , ϵ , θ) (Korpi et al., 2002; Whiting, 2003; Sieghart, 2006). Many GABA-A receptor subtypes contain α , β and γ -subunits with the likely stoichiometry $2\alpha, 2\beta, 1\gamma$ (Korpi et al., 2002; Fritschy and Brunig, 2003). Functional properties of GABA-A receptor depend on its subunit composition, which are differentially expressed both temporally and spatially throughout the brain (Sigel et al., 1990; Pirker et al., 2000; Wohlfarth et al., 2002). Most GABA-A receptors contain a single type of α - and β -subunit variant. The $\alpha_1\beta_2\gamma_2$ hetero-oligomer constitutes the largest population of GABA-A receptors in the brain, followed by the $\alpha_2\beta_3\gamma_2$ and $\alpha_3\beta_3\gamma_2$ isoforms. Receptors that contain the α_4 -, α_5 -, α_6 -, or the β_1 -, γ_1 -, γ_3 -, δ -, ϵ - and θ -subunits are less abundant but play important functions. The α_6 - and δ -subunits in cerebellar granule cells, or the α_4 - and δ -subunits, typically present at extracellular/perisynaptic sites in dentate granule cells and thalamic neurons, mediate tonic current in response to ambient levels of GABA (Mody and Pearce, 2004; Farrant and Nusser, 2005; Walker and Semyanov, 2008). Mutations in certain GABA-A receptor subunits cause epilepsy (DeLorey et al., 1998; Baulac et al., 2001; Macdonald and Kang, 2008) and also alter drug sensitivity (Rudolph et al., 2001; Lambert et al., 2003). A variety of neuroendocrine conditions are associated with profound alterations in GABA-A receptor subunit expression (Smith et al., 1998; Felloso et al., 1998; Maguire et al., 2005).

Analysis of subunit expression is important to understand the pharmacology and functional significance of GABA-A receptor subunits in various regions in the brain. However, studies utilizing traditional RNA assay techniques, such as the RNAase protection assay, the *in-situ* hybridization and the competitive PCR, are tedious, require large quantities of RNA, and often difficult to analyze multiple genes in large sample size (Wisden et al., 1992; Follesa et al., 1998; Liu et al., 2002). The reverse transcription followed by PCR (RT-PCR) is a standard technique routinely used for detecting the gene expression, but its sensitivity is very limited and not amenable for rapid analysis.

Therefore, a more sensitive and reliable technique is required for accurate quantification of GABA-A receptor subunit expression in brain tissue samples. Real-time PCR is a versatile technique for rapid analysis of multiple samples. The use of fluorescent dyes such as SYBR green allows quantitation of the starting amount of nucleic acid by measuring the fluorescence intensity with PCR instrumentation. A non-regulated reference gene such as glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is used as endogenous control for relative analysis of gene expression. The "TaqMan" real-time PCR, which measures PCR-product accumulation during the exponential phase of the PCR reaction using a dual-labelled fluorogenic probe (referred as "TaqMan probe") has been developed and used extensively (Holland et al., 1991; Gibson et al., 1996; Heid et al., 1996; Lie and Petropoulos, 1998). The TaqMan assay is based on the 5'-3' exonuclease activity of *Taq* DNA polymerase to cleave a dual-labeled probe, which is designed to hybridize to a target sequence during amplification (Fig. 1). Disintegration of the probe during PCR releases reporter fluorescence and the intensity of the fluorescence signal measured during the exponential phase of the PCR reaction is proportional to the amount of input target DNA. Unlike the intercalating SYBR green that binds to all double-stranded DNA products, the TaqMan probe avoids detection of non-specific amplification products because of its stringent design to bind to the target gene sequence. The TaqMan assay has been widely used for reliable and sensitive analysis of gene expression of glutamate receptor subunits (Medhurst et al., 2000; Pfaffl, 2001; Horii et al., 2002; Langmann et al., 2003). Studies using the TaqMan technology for GABA-A receptor subunits have appeared, but are restricted to a few of the subunits (Floyd et al., 2004; Linneman et al., 2006; Byrnes et al., 2007). However, there are few studies that describe systematic optimization of TaqMan real-time PCR protocol for GABA-A receptor subunit superfamily.

In this study, we describe the optimization of TaqMan real-time RT-PCR assay for quantification of GABA-A receptor subunit family using the GAPDH as reference gene. The assay was utilized to determine changes in expression of the hippocampal GABA-A receptor α_4 subunit gene, which is known to increase following withdrawal from chronic progesterone and neurosteroids. Our results show that the α_4 -subunit was upregulated threefold following neurosteroid withdrawal in mice.

2. Materials and Methods

2.1. Animals

Female adult mice (25–30 g) of C57BL/6J strain were used in the study. Total RNA extraction was performed from whole brain and hippocampus in untreated adult mice or mice with neurosteroid withdrawal treatment. To induce neurosteroid withdrawal, mice were treated with progesterone (25 mg/kg, s.c., twice daily for 7 days) followed by single injection of finasteride (50 mg/kg, i.p.) on seventh day. Twenty-four hours after finasteride injection, mice were anaesthetized using isoflurane and the hippocampus was rapidly dissected for RNA isolation. Chronic treatment with progesterone is associated with high circulating levels of neurosteroids such as allopregnanolone because progesterone is converted in the brain into allopregnanolone (Reddy et al., 2004). Administration of finasteride, a 5α -reductase inhibitor that blocks conversion of progesterone into allopregnanolone, induces a state of neurosteroid withdrawal. This treatment group was referred to as neurosteroid withdrawal group. The control group received vehicle (15% β -cyclodextrin solution) for 7 days and the brain tissue was collected for RNA isolation. All animal procedures were approved by the Institutional Animal Care and Use Committee.

2.2. Extraction of total RNA

Total RNA was extracted from whole brain and hippocampus using a Trizol reagent from Invitrogen (Carlsbad, CA) as per the manufacturer's instructions. The quality of RNA samples was ascertained by measuring optical density (OD, 260/280) absorption ratio of 1.7 (range 1.62 – 2.1). The integrity of RNA was verified by the detection of 18S and 28S bands after agarose-formaldehyde gel electrophoresis. Total RNA samples were stored at -80°C . To remove residual DNA contamination, the RNA samples were incubated with RNAase-free DNase I at 37°C for 20 minutes, and then the DNase I was inactivated at 65°C for 10min (Ambion, Austin, TX). The purified total RNA was used to generate cDNA.

2.3. Preparation of cDNA

Total RNA from each sample was used to synthesize cDNA using a Superscript II first-strand cDNA synthesis kit (Invitrogen Inc., Carlsbad, CA) with oligo (dT) primers, according to the manufacturer's protocol. Briefly, $2\mu\text{g}$ of DNase-treated total RNA was used as starting material, to which we added $1\mu\text{l}$ of oligo (dT), $1\mu\text{l}$ of 10 mM dNTPs, $2\mu\text{l}$ of $10\times$ first strand buffer, $4\mu\text{l}$ 25 mM MgCl_2 , $2\mu\text{l}$ of 0.1 M DTT, and $1\mu\text{l}$ of RNAase out. The reagents, RNA, oligo (dT), and dNTPs were mixed first, then heated at 65°C for 5 min and then chilled on ice until the other components were added. The samples were incubated at 42°C for 2 min. Then $1\mu\text{l}$ of Superscript II ($40\text{ U}/\mu\text{l}$) was added, and the samples were incubated at 42°C for 50 min. The reaction was inactivated at 70°C for 15 min. The samples were kept on ice, centrifuged briefly, added $1\mu\text{l}$ of RNAse H and incubated at 37°C for 20 min. Parallel reactions for each RNA sample were run in the absence of Superscript II (no RT control) to assess any genomic DNA contamination.

2.4. Design of primers and probes

The PCR primers and TaqMan probe specific for each GABA-A receptor subunit gene and GAPDH were designed using Beacon Designer software (Bio-Rad Inc., Hercules, CA). Like the Primer Express software (Applied Biosystems Inc., Foster City, CA), this program selects probe and primer sets with optimized melting temperatures, secondary structure, base composition, and amplicon lengths. The specificity of the primers and probes for each subunit was confirmed by a homology search. Highly purified salt-free primers were obtained commercially (IDT Inc., Coralville, IA) and TaqMan probes were procured from Applied Biosystems (Foster City, CA). The TaqMan probe was designed with FAM (6-carboxyfluorescein) as the reporter dye on the 5' end and TAMRA (6-carboxytetramethylrhodamine) as the quencher dye on the 3' end (Fig. 1). The parameters for primers and probes were optimized as per the guidelines of the TaqMan assay (Applied Biosystems).

2.5. TaqMan real-time PCR

TaqMan real-time quantitative PCR amplification reactions were carried out in an AB 7500 fast real-time system (Applied Biosystems). Real-time PCR was carried out with TaqMan Universal PCR Master Mix (Applied Biosystems), which contained AmpliTaq Gold DNA Polymerase, AmpErase, UNG, dNTPs with dUTP, and optimized buffer components. AmpErase UNG treatment was used to prevent the possible reamplification of carry-over PCR products. Briefly, a 25- μ L reaction mixture consists of 12.5- μ L TaqMan Universal PCR Master mix, 400 nM primers (a range of 100 – 600 nM were used in optimization studies), 300 nM TaqMan probe (a range of 50–300 nM were used in optimization studies) for each of the target genes. A master mix was prepared, vortexed and aliquoted (24 μ L) into the wells of a 0.2-mL optical-grade 96-well PCR plate (Applied Biosystems). Then, 1 μ L of cDNA template was added to a final volume of 25 μ L. All reactions were carried out in triplicate with no template control as well as no-RT sample. The thermal cycle conditions were: 50 °C for 2 min (*AMPerase activation*), 95 °C for 10 min (*Taq activation*), 95 °C for 15 sec for denaturation, and 60 °C for 1 min for annealing and extension. The AB 7500 system measures a fluorescent accumulation of PCR product by continuous monitoring. The program was set to monitor the complete amplification for 50 cycles. All TaqMan PCR data were captured using the SDS software (Applied Biosystems). For every sample, an amplification plot was generated showing the increase in the reporter dye fluorescence (Rn) with each cycle of PCR. Quantification of input target amount was analyzed by cycle threshold (C_T) value, the point at which the sample PCR amplification plot crosses the threshold.

2.6. Optimizing primer and probe concentrations

For each target gene, the optimum primer and probe concentration was determined with a fixed template level (10^4 copy numbers). A combination of four concentrations (100 – 600 nM) of both forward and reverse primers with a constant probe concentration was determined. The combination showing the highest fluorescence was tested thereafter at four different TaqMan probe concentrations (50 – 300 nM). The combination of primer and TaqMan probe concentration that yielded optimal assay performance was chosen for further experiments.

2.7. Generation of calibration curves

A calibration curve approach was used to establish the results obtained by the TaqMan PCR assay. For this purpose, we prepared mouse whole brain cDNA using purified total RNA. PCR products were generated for each target subunit with specific primers using mouse whole brain cDNA as template. The specificity of PCR products generated for each subunit was confirmed by agarose-gel electrophoresis and ethidium bromide staining. The single bands were excised from the gel and eluted using the gel extraction kit (Quiagen, Valencia, CA). PCR products

were sequence verified by sequence service center (MWG, High Point, NC). Each target subunit PCR product was quantified using spectrophotometer. Copy number (C , copies/ μ l) for each subunit template was calculated using the following equation: $C = PA/GMX$, where P is the PCR product concentration (ng/ μ l), A is Avogadro's constant (6×10^{23}), G is the amplicon size (bp), M is the multiplying constant (1×10^9) and X is the average molecular weight of the 1 bp in the amplicon (650 Daltons). Copy numbers for each target subunit were serially diluted from 10^{10} to 10^1 copies and were used as template for TaqMan assay. A calibration curve was plotted for each target subunit and GAPDH using known copy number verses C_T values.

2.8. Data analysis

Linear regression and correlation analysis of real-time PCR amplification data was performed using SDS software (Applied Biosystems). Standard curves for each subunit gene were plotted showing C_T value versus log of initial copy number of cDNA. The slope of the standard curve was calculated to describe the efficiency of PCR (if the PCR amplification is exponential, resulting in a doubling of product in every cycle, the slope will be -3.3 as 3.3 cycles are required to generate a 10-fold increase in product). The GABA-A receptor subunit expression was analyzed based on the relative quantification approach as outlined in AB 7500 User Bulletin (Applied Biosystems). The relative quantification provides accurate change in expression of the target gene in the experimental sample relative to a control calibrator sample. The initial target input amount for each target subunit and GAPDH were calculated using standard curve method. The quantity calculated for target subunit was normalized with GAPDH as the endogenous control. The normalized value of control (vehicle group) was taken as the calibrator to calculate the relative expression levels of target genes in the experimental group. The target gene expression in the samples was expressed as percent change from control. The unpaired t -test was used to compare the effect of neurosteroid withdrawal on $\alpha 4$ subunit expression in mice. The criterion for statistical significance was $p < 0.05$.

3. Results

3.1. Specificity of TaqMan probe and primers

The TaqMan probe and primer pair designed for GABA-A receptor subunit genes are listed in Table 1. Specificity of primers and probes were verified by homology search. To confirm the specificity of primers for each target subunit, a traditional RT-PCR followed by agarose gel electrophoresis was performed using mouse whole brain cDNA as a template. As shown in Fig. 2, a single PCR product with the desired length was observed for each target subunit gene and the reference GAPDH. No band was observed in the negative control samples without RT (data not shown). To ascertain the possible contamination or non-specific products in the PCR reaction, all primer pairs were also tested with no template cDNA as negative control that showed no band (Fig. 2). Moreover, nucleotide sequence analysis of the RT-PCR products revealed an identical sequence to each target GABA-A receptor subunit amplicons as listed in Table 1. Therefore, the probe and primers pairs were highly specific to target genes and do not produce non-specific amplicons.

3.2. Amplification plots and sensitivity of TaqMan reactions

Amplification plots were derived for GABA-A receptor subunits (α_{1-6} , β_{1-3} , γ_2 , and δ) and GAPDH using increased template copy numbers ranging from 10^2 to 10^9 . A representative amplification plot of the α_2 -subunit was shown in Fig. 3. The plot shows that fluorescence (R_n) increases with increased copy number of template cDNA in the samples. The samples with highest target subunit template were detected earlier in the PCR cycles as shown by the leftward shift of the amplification plot. Amplification plots show that the TaqMan assay was highly sensitive to detect very low levels of gene expression (~ 100 copies of the template cDNA). The amplification plots provided two valuable parameters – threshold (a level of delta R_n that

was automatically set to be above the baseline) and cycle threshold, also known as C_T value (the fractional cycle number at which the fluorescence passes the threshold). Quantification of input target amount was analyzed by C_T value, which in turn predicts the quantity of input target cDNA. The C_T value was utilized for construction of standard curves and data analysis in the TaqMan assay.

3.3. Optimization of primers and probe concentrations

We optimized the probe and primer concentrations by performing a series of experiments with varying primer-probe combinations. Figure 4 shows the amplification plots for all primer concentration combinations for the GABA-A receptor α_1 subunit and GAPDH. The C_T values derived from these plots were listed in Table 2. The combination of 400 nM forward and reverse primer combination showed a plot with both the highest fluorescence and lowest C_T value. At this primer concentration, the probe combination of 300 nM yielded a plot with both highest fluorescence and lowest C_T value (Table 2). Further experiments were carried out using a combination of 400 nM primers and 300 nM probe concentration.

3.4. Selection of endogenous reference gene

To quantify levels of target mRNA expression, the GAPDH was used as an endogenous reference gene for relative analysis of gene expression. A specific set of primers and probe was designed for mouse GAPDH gene and the levels of GAPDH mRNA in samples from control and experimental groups were determined by TaqMan real-time PCR. Data from extensive series of experiments shows that C_T values were similar in different groups (C_T values ranged 17–18), indicating that GAPDH expression was found to be unaffected by treatments.

3.5. Standard curves of GABA-A receptor subunit genes

To determine the PCR efficiency and linearity of template amplification, standard curves were constructed for each of the GABA-A receptor subunit genes and GAPDH using mouse whole brain cDNA as template. As illustrated in methods, known copy numbers calculated from the purified PCR products ranging from 10^2 to 10^8 copies of the templates were used. The TaqMan assay was performed in triplicate for 50 cycles and C_T values obtained were plotted against the log cDNA copy numbers. Standard curves derived for α_1 , α_2 , α_4 , and α_6 subunit and GAPDH were shown in Fig. 5. Standard curves for other GABA-A receptor subunits (β_2 , β_3 , γ_2 , and δ) were shown in Fig. 6. A reverse linear relationship was detected over four orders of magnitude for each of the target subunits and GAPDH. PCR amplification efficiency was assessed for each primer and probe set from the slope of the standard curve. In most cases, the slope of the standard curve was close to -3.4 , indicating maximal PCR amplification efficiency. The C_T values and cDNA template concentrations show a reverse linear correlation with nearly identical slopes. The correlation coefficients were in the range of 0.990 to 0.998, indicating the accuracy of the amplification efficiency.

3.6. Effect of neurosteroid withdrawal on α_4 subunit expression

The optimized protocol for TaqMan assay was utilized to determine changes in the expression of the hippocampal GABA-A receptor α_4 subunit gene, which is known to significantly upregulate in animals undergoing withdrawal from chronic progesterone or neurosteroid treatment. Mice were treated chronically with progesterone to induce high levels of neurosteroids and then withdrawn by finasteride injection to inhibit biosynthesis of the neurosteroid allopregnanolone. As shown in Fig. 7B, the α_4 -subunit expression was increased significantly (~ 3 -fold) in the hippocampus following neurosteroid withdrawal in mice. The absolute expression, represented as the mRNA copy number, was found to be similar and consistent with the pattern of change noted in relative expression using GAPDH as an internal control (Fig. 7A). There was no change in expression levels of GAPDH following neurosteroid

withdrawal (control: $205,937 \pm 9,868$ copy numbers; withdrawal: $210,062 \pm 29,339$ copy numbers). These results are consistent with the previous studies demonstrating that progesterone or neurosteroid withdrawal increases the α_4 subunit expression in the hippocampus (Smith et al., 1998; Gulinello et al. 2001).

4. Discussion

The present study shows successful optimization of TaqMan real-time RT-PCR assay for analysis of GABA-A receptor subunit expression in brain tissues. The key findings of this study are: (i) The TaqMan technology is optimized for quantification of mouse GABA-A receptor α_{1-6} , β_{1-3} , γ_2 and δ subunits; (ii) The primers and probes used in the assay selectively amplify the specific target gene as verified by the electrophoresis of PCR products and sequencing of the products; (iii) The TaqMan assay is highly sensitive and can detect very low levels of gene expression (~10 to 100 copies of template mRNA), and thus requires very small amount of mRNA (low ng levels) compared to traditional RNA assays; (iv) The TaqMan assay is superior in specificity and accurately measures target subunit expression using the GAPDH as internal control; and (v) The TaqMan assay is effectively utilized by confirming the neurosteroid withdrawal-induced upregulation of the α_4 -subunit expression.

The real-time PCR is a versatile tool that can be optimized for enhanced sensitivity, high throughput analysis, and minimal post-PCR manipulations. Two types of fluorescent reagents (SYBR green dye and TaqMan probe) allow for real-time monitoring of the PCR. The SYBR green method is widely used to measure the fluorescence of amplified products in the real-time quantitative PCR assay (Gutala and Reddy, 2004). The SYBR green assay is a less-expensive alternative to TaqMan assay. However, SYBR green chemistry is less specific and can detect non-specific amplification products because of its intercalating mechanism. Since SYBR green nonspecifically detects all double-stranded DNA, it is not possible to distinguish between the specific amplified product and the primer-dimers formed during the reaction. Unlike SYBR green technology, the TaqMan assay is highly specific because the probe is designed for highly specific hybridization with target cDNA sequences. The TaqMan PCR is more suitable for transcriptional profiling of GABA-A receptor subunit plasticity because of several closely related subunits.

In the present study, we optimized primers and probes for mouse GABA-A receptor subunit family (Table 1). The sequences are retrieved from NCBI web site. Primers and probes are designed for each subunit gene using optimum parameters according to the guidelines described for real-time PCR. In the design of TaqMan probes, we considered several important factors such as T_m , the presence at 3' end of the primer of A's or T's, and shorter amplicon size. Primers for each subunit are chosen to select from two different exons. This type of intron flanking primer design distinguishes the amplicons by their size from genomic DNA contamination. The γ_2 subunit has two splice variants such as γ_{2L} , γ_{2S} which differ by 24bp. Primers and probes for γ_2 subunit are designed from a common sequence for both splice variants, so that in these one would recognize expression of both the splice variants. Primers for all subunit genes are validated by performing regular RT PCR using mouse whole brain cDNA as a template. The specificity of primers is confirmed by a single band with correct size amplicon detected on agarose gel-electrophoresis. These PCR products are gel purified and further confirmed by DNA sequencing. Thus, the TaqMan PCR offers additional sensitivity because of the dual-labeled probe, which is specific for each target subunit. This is a highly desirable feature when analyzing a complete superfamily of GABA-A receptor subunits that have multiple closely related homologous variants.

In real-time PCR assays, a quantitative relationship exists between amount of input target template and amount of PCR product at any given cycle number. Therefore, amplification of

the target gene at high efficiency over dynamic input ranges is important for accurate quantitation of gene expression. In the present study, a standard curve for GAPDH and each target gene is constructed ($10^2 - 10^9$ copy numbers of template cDNA). Purified PCR products generated using the specific primers of each target subunit are used as a template to generate standard curves. The standard curve demonstrated excellent correlation coefficient ranging from 0.990 to 0.998 indicating accurate amplification of each target subunit gene. The slopes for GAPDH and target genes are around -3.4 , reflecting similar optimum PCR efficiencies. The template concentrations are prepared with suitable dilution so that the input levels can be detected from the standard curves. It is possible that the amplification may not be equally scaled at certain dilutions, especially lower dilutions (higher copy numbers). However, such exceptions do not appear to affect the amplification of target subunits because standard curves are highly consistent with ideal amplification of target subunits with slopes around -3.4 ($r^2=0.99$). Amplification plots can be further optimized using alternative primers and probe for maximum efficiency and specificity.

The present study utilized the relative expression approach for quantification of target gene expression. Generally two quantification strategies can be utilized in real-time PCR: an absolute and a relative quantification. In absolute quantification, the absolute mRNA copy number is determined by comparison to appropriate external calibration curve (Pfall, 2001; Pfaffl and Hageleit, 2001). The relative expression is based on the expression levels of a target gene versus a reference gene and is adequate for most purposes to investigate physiological changes in gene expression levels. In this study, GAPDH is chosen as a reference gene. Reliable endogenous controls are critical for achieving accurate results in real-time PCR. Ideally, the endogenous control should not be altered in the disease or under treatment conditions, and should be expressed roughly the same level as the RNA under study (Thellin et al., 1999; Bustin, 2000). GAPDH is the most widely used reference gene (Uno et al., 2002; Horii et al., 2002). In the present study, the expression of GAPDH is not altered under the neurosteroid withdrawal conditions. Alternative reference genes such as β -actin, β_2 -microglobulin or 18S RNA or other unregulated housekeeping genes can be considered if the GAPDH is likely to change under the experimental conditions (Gutala and Reddy, 2004; Floyd et al., 2004). There are several advantages of using more than one housekeeping gene for the purpose of data normalization such as avoiding false positive changes and accurate detection or expression of fold change in target gene expression.

The TaqMan assay for GABA-A receptor subunit expression can be used to accurately measure changes in subunit expression under various physiological or pharmacological conditions. Progesterone exposure and withdrawal has been shown to distinctly affect GABA-A receptor subunit plasticity (Follesa et al., 1998; Reddy, 2004). The most striking finding following withdrawal from progesterone or the neurosteroid allopregnanolone is the marked up regulation of the α_4 -subunit in the hippocampus (Smith et al., 1998; Gulinello et al., 2001). It has been suggested that the increased expression of the α_4 - subunit might decrease the net GABA-A receptor-mediated inhibition and promote excitability leading to increased seizure exacerbation (Reddy, 2004). Most of those subunit plasticity studies utilized conventional RNA assays (Wisden et al., 1992; Follesa et al., 1998; Liu et al., 2002) or Western blots (Sun et al., 2004; Maguire et al., 2005). Despite some reports using real-time PCR (Linneman et al., 2006; Byrnes et al., 2007), a detailed protocol describing TaqMan methodology for GABA-A receptor subunits is lacking. In the present study, the TaqMan assay is used to determine the effect of neurosteroid withdrawal on GABA-A receptor α_4 - subunit expression in the hippocampus in mice. We found that α_4 - subunit is upregulated significantly following neurosteroid withdrawal. These results are highly consistent with previous reports on α_4 - subunit expression (Smith et al., 1998; Gulinello et al. 2001).

The TaqMan RT-PCR has several advantages over conventional RT-PCR methods. The TaqMan PCR is a “closed-tube” homogenous system avoiding time-consuming and hazardous post-PCR processing and decreasing the potential risk of contamination. The TaqMan PCR allows precise and reproducible quantification because it is based on C_T values rather than end-point detection, where the PCR components are rate limiting. The amount of cDNA template required (1 to 25 ng) is much lower than the amount needed for the conventional RNA assays such as Northern blot analysis (μg). This method is very useful for analysis of GABA-A receptor subunits in single cells or in samples from single RNA pool with low copy number of the subunit genes. Other advantages include the availability of automated real-time PCR systems that rapidly monitor a large number of samples in a 96- or 384-well format that permits a high-throughput screening of large sample size.

In conclusion, these results suggest that the real-time TaqMan RT-PCR assay is a precise, reliable, and sensitive method for quantification of GABA-A receptor subunits in brain tissues. This TaqMan assay allows rapid, high-throughput profiling of complete GABA-A receptor superfamily, and thus provides a powerful tool for investigations of GABA-A receptor subunit plasticity in animal models of neurological and psychiatric conditions.

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Fig. 1. Diagrammatic illustration of the TaqMan chemistry

The TaqMan PCR reaction exploits the 5'-nuclease activity of Taq DNA polymerase to cleave a TaqMan probe during PCR. The TaqMan probe is an oligonucleotide that contains a reporter dye (FAM, 6-carboxytetramethylrhodamine) at the 5'-end of the probe and a quencher dye (TAMRA, 6-carboxyfluorescein) at the 3'-end of the probe. During PCR, the probe specifically anneals to the target sequence between the forward and reverse primer sites. When the probe is intact, the quencher dye suppresses the fluorescence emission of the reporter dye primarily due to Forster-type energy transfer. During the PCR reaction, Taq DNA polymerase extends the primer through the polymerase activity, as it approaches the probe it displaces the probe and cleaves it through the 5' to 3' exonuclease activity. This separates the reporter dye and the quencher dye from the probe, which results in increased fluorescence of the reporter. Accumulation of PCR products is detected in "real-time" directly by monitoring the increase in fluorescence of the reporter dye with an automated PCR system.

Fig. 2. Gel electrophoresis of PCR products of GABA-A receptor subunits and GAPDH

The target subunits and GAPDH gene expression from mouse whole brain cDNA were analyzed by RT-PCR using specific primers. A single band was detected near the expected size for each target subunit and GAPDH. No band was observed in the PCR products of negative control samples without template DNA. In the panel (M) indicates 100 bp molecular weight marker, (-) indicates PCR reaction with no cDNA template, (+) indicates PCR reaction with template cDNA.

Fig. 3. Amplification plots of the GABA-A receptor α_2 -subunit in the TaqMan real-time PCR assay TaqMan assay for the GABA-A receptor α_2 subunit was performed using purified PCR product generated with specific primers and the template sample was serially diluted 10-fold from 10^9 to 10^2 copy numbers. The fluorescence emission measured R_n (reporter normalized fluorescence) was plotted against cycle number. In the amplification plots, fluorescence intensity increased as the PCR cycles increased. Negative control sample (no template DNA sample) showed no elevation of fluorescence. The legends (log 2 to 9) refer to the copy number of the template DNA used. TaqMan assay for GAPDH was performed similarly using purified PCR product serially diluted 10 fold from 10^8 to 10^2 copy numbers (data not shown). The threshold cycle (C_T), the point at which the fluorescence exceeds a threshold limit is used as key parameter for calculating input cDNA for relative quantification of the target subunit expression.

Fig. 4. Optimization of primer and TaqMan probe concentrations in the TaqMan assay
 Primer concentration-dependent amplification plots of GAPDH (A) and the GABA-A receptor α_1 -subunit (B). Forward and reverse primers were tested at four different concentrations (100 – 600 nM) with a fixed probe concentration (300 nM) using whole brain cDNA for GAPDH and α_1 -subunit. The primer pair yielding the best amplification (400 nM) was chosen to determine the optimum probe concentration. TaqMan probe was tested at four different concentrations (50 – 300 nM) for GAPDH and α_1 -subunit and the C_T values derived from the amplification plots are listed in Table 2. For most target subunits, 300 nM of probe yielded the best amplification with lowest C_T values. The curves represent the mean of triplicate measurements.

Fig. 5. Standard curves for GAPDH and selected GABA-A receptor subunits

Standard curves for GABA-A receptor α_1 (A), α_2 (B), α_4 (C), and α_6 subunit (D). Standard curves for each subunit and GAPDH were constructed using log cDNA copy number against C_T values. The C_T values and sample concentrations show a reverse linear correlation with nearly identical slopes of around (-3.4) , indicating equivalent PCR efficiencies of target and GAPDH control. The correlation coefficient for standard curves ranged from 0.990 to 0.998, suggesting the accuracy of quantification protocol. Each point represents the mean of triplicate measurements for each subunit.

Fig. 6. Standard curves for GAPDH and selected GABA-A receptor subunits

Standard curves for GABA-A receptor γ_2 (A), δ (B), β_2 (C), and β_3 subunit (D). Standard curves for each subunit and GAPDH were constructed using log cDNA copy number against C_T values. The C_T values and sample concentrations show a reverse linear correlation with nearly identical slopes of around (-3.4) , indicating equivalent PCR efficiencies of target and GAPDH control. The correlation coefficient for standard curves ranged from 0.990 to 0.998. Each point represents the mean of triplicate measurements for each subunit.

Fig. 7. Changes in the GABA-A receptor α_4 -subunit expression in the hippocampus following neurosteroid withdrawal in mice

The upper panel (A) shows the absolute expression of α_4 mRNA in terms of copy numbers, while the relative expression was shown in the lower panel (B) as the percent change in the ratio of α_4 /GAPDH copy numbers in each animal. The α_4 -subunit was up-regulated (~3-folds) in the hippocampus of mice chronically treated first with progesterone (25 mg/kg, sc, twice daily for 7 days) and then with finasteride (50 mg/kg, ip on day 7) which produces withdrawal of the neurosteroid allopregnanolone by inhibiting its synthesis from progesterone. Hippocampus was isolated 24 h after finasteride injection, total RNA was extracted and cDNA prepared from vehicle and neurosteroid withdrawal groups for α_4 -subunit analysis. The data

was normalized in every assay using the internal GAPDH gene, which was not significantly altered following neurosteroid withdrawal. Data represent mean $\pm$ SEM (n = 8 mice per group). *p<0.01 versus control group.

TABLE 1
Sequences of primers and TaqMan probes for mouse GABA-A receptor subunits.

Gene	Primer/Probe Sequence	Position	Size	Amplicon
<i>α-1 (accession # NM_010250)</i>				
Forward:	5'-CAA GAG CAG AAG TTG TCT ATG AGT-3'	1346	24	215 bp
Reverse:	5'-GCA CGG CAG ATA TGT TTG AAT AAC-3'	1560	24	
Probe:	5'-6-FAM-ACC AGA GAG CCA GCC CGT TCA GTG-TAMRA-3'	1372	24	
<i>α-2 (accession # NM_008066)</i>				
Forward:	5'-GCT ACG CTT ACA CAA CCT CAG A-3'	848	22	117 bp
Reverse:	5'-GAC TGG CCC AGC AAA TCA TAC T-3'	964	22	
Probe:	5'-6-FAM-ACC TGG AGC CAT CGG GAG CAA CCT-TAMRA-3'	934	24	
<i>α-3 (accession # NM_008067)</i>				
Forward:	5'-GCC GTC TGT TAT GCC TTT GTA TTT -3'	1242	24	118 bp
Reverse:	5'-TTC TTC ATC TCC AGG GCC TCT -3'	1360	21	
Probe:	5'-6-FAM-TGC CTT CCC AAG CCC AAC TCC GC -TAMRA-3'	1329	23	
<i>α-4 (accession # NM_010251)</i>				
Forward:	5'-AGA ACT CAA AGG ACG AGA AAT TGT -3'	606	24	118 bp
Reverse:	5'-TTC ACT TCT GTA ACA GGA CCC C -3'	723	22	
Probe:	5'-6-FAM-ACG CAG CCT GTT GTC ATA ACC ATC CAG C-TAMRA-3'	691	28	
<i>α-5 (accession # NM_176942)</i>				
Forward:	5'-AAG TTC GCT CCG GCA GTA TG -3'	150	20	148 bp
Reverse:	5'-TGT TCT TGC CTC CAA CTT GAT CT -3'	298	23	
Probe:	5'-6-FAM- AAG TTC GCT CCG GCA GTA TG – TAMRA-3'	178	20	
<i>α-6 (accession # NM_001099641)</i>				
Forward:	5'-CTT GCT GGA AGG CTA TGA CAA C -3'	397	22	146 bp
Reverse:	5'-AAG TCT GGC GGA AGA AAA CAT C -3'	542	22	
Probe:	5'-6-FAM-ACT CCA TCT CCA CAT CTG ACA CAG GTC CA-TAMRA-3'	512	29	
<i>β-1 (accession # NM_008069)</i>				
Forward:	5'-TAT TCT CCT CAG CAC CCT GGA A -3'	1098	22	90 bp
Reverse:	5'-CGA GTA CAT GGT GGC CTT GG -3'	1188	20	
Probe:	5'-6-FAM-CAC CTG TGA GCA CCT CGG AGC CA-TAMRA-3'	1160	23	
<i>β-2 (accession # NM_008070)</i>				
Forward:	5'-AAC TAC ATC TTC TTT GGG AGA GGA -3'	1406	24	106 bp
Reverse:	5'-GGT CCA TCT TGT TGA CAT CCA G -3'	1511	22	
Probe:	5'-6-FAM-CGC ATC TTC TCA TTG TTG GCA TTA GCA GC-TAMRA-3'	1488	29	
<i>β-3 (accession # NM_008071)</i>				
Forward:	5'-CTT TGC GGG AGG AAG GCT TT-3'	112	20	85 bp
Reverse:	5'-GGG GTC GTT TAC GCT CTG AG -3'	196	20	
Probe:	5'-6-FAM-AGC AAA CCA CCG CCA CCA GCA CC-TAMRA-3'	173	23	
<i>γ-1 (accession # NM_010252)</i>				
Forward:	5'-GTA AAA GCG AAC CCG ATG GG -3'	384	22	123 bp
Reverse:	5'-CGA CAC AGT TTC CCA GAT GC -3'	507	20	

Gene	Primer/Probe Sequence	Position	Size	Amplicon
Probe:	5 '-6- FAM- AGC CTT CCT CTT CTC TCC TTC TCT CCT GTG-TAMRA-3'	413	30	
<i>γ-2 (accession # NM_010252)</i>				
Forward:	5'-ACT TCT GGT GAC TAT GTG GTG AT -3'	1108	23	147 bp
Reverse:	5'-GGC AGG AAC AGC ATC CTT ATT G -3'	1254	22	
Probe:	5'-6-FAM-AAG GAC ACC CAG GAC AGG ACC ACG A-TAMRA-3'	1226	25	
<i>γ-3 (accession # NM_008074)</i>				
Forward:	5'-GGATTCCAGACACCATCTTTCGA -3'	478	23	93 bp
Reverse:	5'-TTCCCATCATTCAGATTCTGAGG -3'	571	24	
Probe:	5'-6-FAM-ACAGCGGAGGCTCACTGGATCACC - TAMRA-3'	510	24	
<i>δ (accession # NM_008072)</i>				
Forward:	5'-GGA CGT TCT GGG CTG GC -3'	110	17	146 bp
Reverse:	5'-CCT CCA TTA AGC CAT CCA GGT-3'	255	21	
Probe:	5'-6-FAM-CTG CTG CCG CTC CTG CTG CTC T-TAMRA-3'	129	22	
<i>GAPDH (accession # NM_008084)</i>				
Forward:	5'-CAA TGT GTC CGT CGT GGA TCT -3'	754	21	124 bp
Reverse:	5'-GTC CTC AGT GTA GCC CAA GAT G-3'	877	22	
Probe:	5'-6-FAM-CGT GCC GCC TGG AGA AAC CTG CC-TAMRA-3'	777	23	

TABLE 2
 C_T values of GAPDH and the GABA-A receptor α_1 -subunit derived from amplification plots with various primer and probe combinations.

Primer concentration	Probe concentration	C_T value
GAPDH		
100 nM/100 nM	300 nM	28.0
200 nM/200 nM	300 nM	27.8
400 nM/400 nM	300 nM	27.5
600 nM/600 nM	300 nM	27.7
400 nM/400 nM	50 nM	29.4
400 nM/400 nM	100 nM	28.5
400 nM/400 nM	200 nM	28.2
400 nM/400 nM	300 nM	27.9
α_1-Subunit		
100 nM/100 nM	300 nM	32.14
200 nM/200 nM	300 nM	31.64
400 nM/400 nM	300 nM	31.26
600 nM/600 nM	300 nM	31.20
400 nM/400 nM	50 nM	33.32
400 nM/400 nM	100 nM	32.24
400 nM/400 nM	200 nM	31.55
400 nM/400 nM	300 nM	31.50

The data is the mean of triplicate measurement for each subunit.