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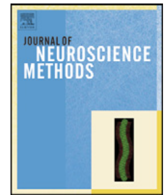

The optimization of TaqMan real-time RT-PCR assay for transcriptional profiling of GABA-A receptor subunit plasticity

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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.

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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 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 2α,2β,1γ (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; Follesa 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,

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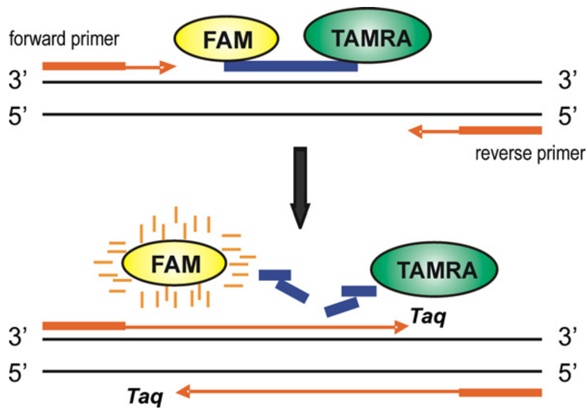

Fig. 1. Diagrammatic illustration of the TaqMan chemistry.
The TaqMan PCR reaction exploits the 5′-nuclease activity of Taq DNA polymerase to cleave a TaqMan probe during PCR. The TaqMan probe is an oligonucleotide that contains a reporter dye (FAM, 6-carboxytetramethylrhodamine) at the 5′-end of the 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, and 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.

require large quantities of RNA, and often its 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-labeled 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; Linnemann 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 min, and then the DNAase I was inactivated at 65 °C for 10 min (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 μg of DNAse-treated total RNA was used as starting material, to which we added 1 μl of oligo (dT), 1 μl of 10 mM dNTPs, 2 μl of 10× first strand buffer, 4 μl 25 mM $MgCl_2$, 2 μl of 0.1 M DTT, and 1 μl 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 μl of Superscript II (40 U/μl) 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 μl 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.

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