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BRIEF METHOD

Activity-Induced Expression of Common Reference Genes in Individual CNS Neurons

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The quantitative study of gene expression in neurons under physiological and pathophysiological conditions has gained considerable importance. Consequently, a variety of different techniques to quantify gene expression have evolved. Generally, such approaches can be subdivided into strategies that provide absolute versus relative quantitation of a target transcript. Quantitative procedures such as quantitative Northern blots or conventional quantitative reverse transcription polymerase chain reaction (RT-PCR) methods have several disadvantages. For instance, Northern blots require substantial amounts of RNA, rendering investigation of mRNA levels from single cells impossible. Comparability of results is extremely dependent upon identical amounts of starting mRNA used in different samples, a condition that cannot usually be achieved with small cell numbers or minute amounts of tissue. To circumvent these obstacles, various techniques have been developed to quantify gene expression relative to a reference gene (Fink et al, 1998; Waha et al, 1998). This obviates the need to perform an accurate determination of initial mRNA concentrations. On the other hand, a suitable reference gene is essential as an internal control for relative mRNA quantitation. This internal control is crucial for a quantitative comparison of gene expression between different tissue types, at various developmental stages, and between control and disease tissue. Ideally, internal control transcripts should show constant, high-level expression independent of experimental conditions. The genes for glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) and *β-actin* have been commonly used as reference genes. In recent years, *cyclophilin A* and hypoxanthine phosphoribosyl-transferase (*HPRT*) have also been adopted for this purpose. However, several classical reference genes may exhibit considerable changes in expression in cell types or under disease conditions (Suzuki et al, 2000). Here, we have analyzed several of these genes as reference transcripts for activity-dependent chronic changes in central nervous system (CNS) gene expression. Pilocarpine-induced epilepsy in rats was employed as experimental paradigm. We find that *GAPDH* is not suitable for this purpose, whereas the neuron-specific gene *synaptophysin* appears remarkably stable following intense neuronal activity.

To study activity-dependent alterations in reference gene expression, seizure activity was induced in vivo by applying a single dose of the convulsant pilocarpine (male Wistar rats 150–200 g, 300–380 mg/kg, ip) after prior administration of methyl-scopolamine (1 mg/kg, sc, 30 minutes before pilocarpine, ip). Seizures were terminated after 2 hours by application of diazepam (0.1 mg/kg, sc). Age-matched rats, injected with saline served only as controls. Thirty days after pilocarpine/saline injection, the brains of pilocarpine-treated and control animals ($n = 5$ each) were removed. One hippocampus was immediately frozen in liquid nitrogen for in situ hybridization, whereas the contralateral hippocampus was used for preparation of 400-μm transverse vibratome slices. From these slices, single CA1 neurons were harvested with a glass pipette in less than 10 μl of extracellular solution (Beck et al, 1999). mRNA was isolated using the Dynabead mRNA Direct Micro Kit (Dynal, Hamburg, Germany) according to the manufacturer's protocol.

For real-time RT-PCR fluorescent hybridization, oligonucleotides and primers were chosen for the five putative reference genes: *GAPDH*, *β-actin*, *cyclophilin A*, *HPRT*, and *synaptophysin* (Eurogentec, Seraing,

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Table 1. Sequences and Concentration Ratios of Primers and Hybridization Probes with Labeling Dyes Used for RT-PCR

Gene	Forward (f)/reverse (r) primer	Hybridization probe[a]	Primer concentrations (nM)(f/r)
GAPDH	(f), 5′-TGCCAAGTATGATGACATCAAGAAG-3′ (r), 5′-TAGCCCAGGATGCCCTTTAGT-3′	F5′-TGGTGAAGCAGGCGGCCGAG-3′T	50/300
Synaptophysin	(f), 5′-TCAGGACTCAACACCTCAGTGG-3′ (r), 5′-AACACGAACCATAAGTTGCCAA-3′	F5′-TTTGGCTTCCTGAACCTGGTGCTCTG-3′T	300/300
β-actin	(f),5′-AGGCCCCTCTGAACCCTAAG-3′ (r), 5′-CCAGAGGCATACAGGGACAAC-3′	F5′-TTTGAGACCTTCAACACCCCAGCCA-3′T	300/300
HPRT	(f), 5′-GCGAAAGTGGAAAAGCCAAGT-3′ (r), 5′-GCCACATCAACAGGACTCTTGTAG-3′	F5′-CAAAGCCTAAAAGACAGCGGCAAGTTGAA-3′T	300/900
Cyclophilin A	(f), 5′-CCCACCGTGTTCTTCGACAT-3′ (r), 5′-AAACAGCTCGAAGCAGACGC-3′	F5′-ACGGCTGATGGCGAGCCCTTG-3′T	50/300

RT-PCR, reverse transcription polymerase chain reaction; *GAPDH*, glyceraldehyde-3-phosphate dehydrogenase; *HPRT*, hypoxanthine phosphoribosyl-transferase.
[a] F, FAM, is the reporter dye; T, TAMRA, is the quencher dye.

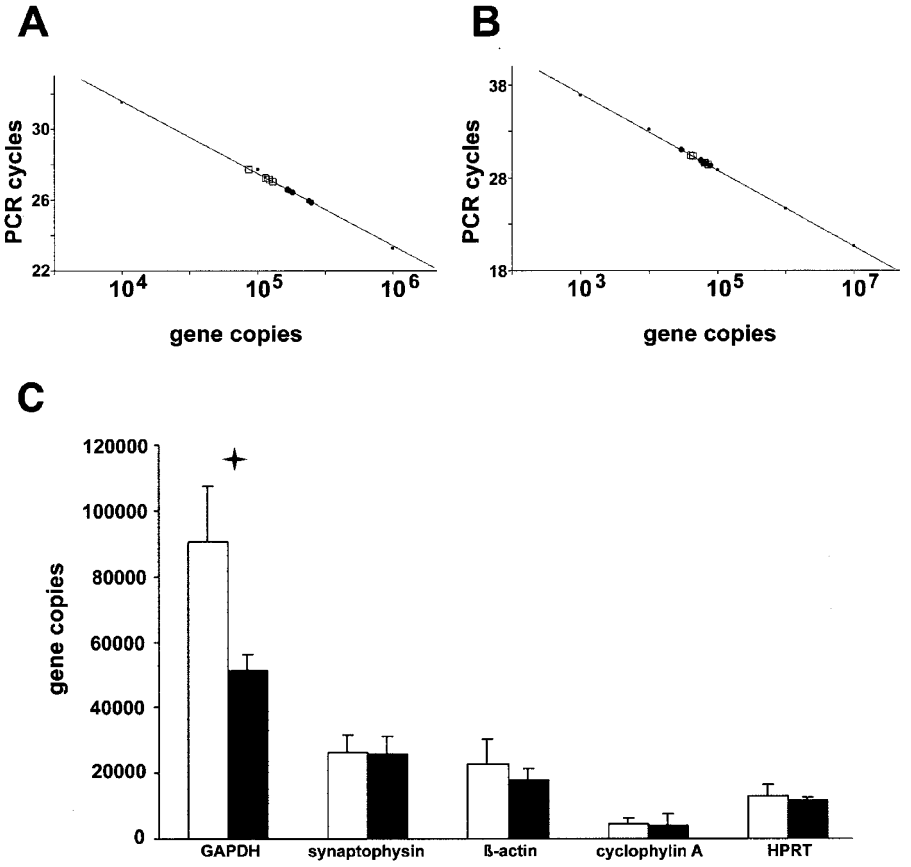

Figure 1.
Representative standard curves for *GAPDH* (A) and *synaptophysin* (B) expression levels. For *GAPDH*, control (*empty squares*) and pilocarpine-treated animals (*filled circles*) form two expression clusters (A). *Synaptophysin* expression is not significantly different between control (*empty squares*) and pilocarpine-treated animals (*filled circles*) (B). Standards are presented as *filled squares*. C, Quantification of reference genes in pilocarpine (white) vs control (black) groups.

Belgium) (Table 1). RT-PCR reactions were performed in the ABI Prism 7700 SDS using TaqMan EZ RT-PCR core reagents (Perkin Elmer, Foster City, California). Single reactions contained 1.5 μl of mRNA, 1× TaqMan EZ Buffer, 5.0 mM $Mn(OAc)_2$, 200μM each of deoxyribonucleoside triphosphate (dNTP), primers (Table 1), 100 nM TaqMan probe, 0.01U/μl AmpErase UNG, and 0.1U/μl rTth polymerase in a volume of 12.5 μl. Amplification conditions were 2 minutes at 50° C, 20 minutes at 60° C, and 5 minutes at 95° C, followed by 60 cycles at 94° C for 15 seconds and 59° C for 1 minute. Fluorescent signals were normalized to a passive internal reference (ΔRn) and a threshold cycle (C_t) determined where ΔRn reaches 10 times standard deviation of the baseline. For absolute quantitation of target transcripts, standard curves were generated

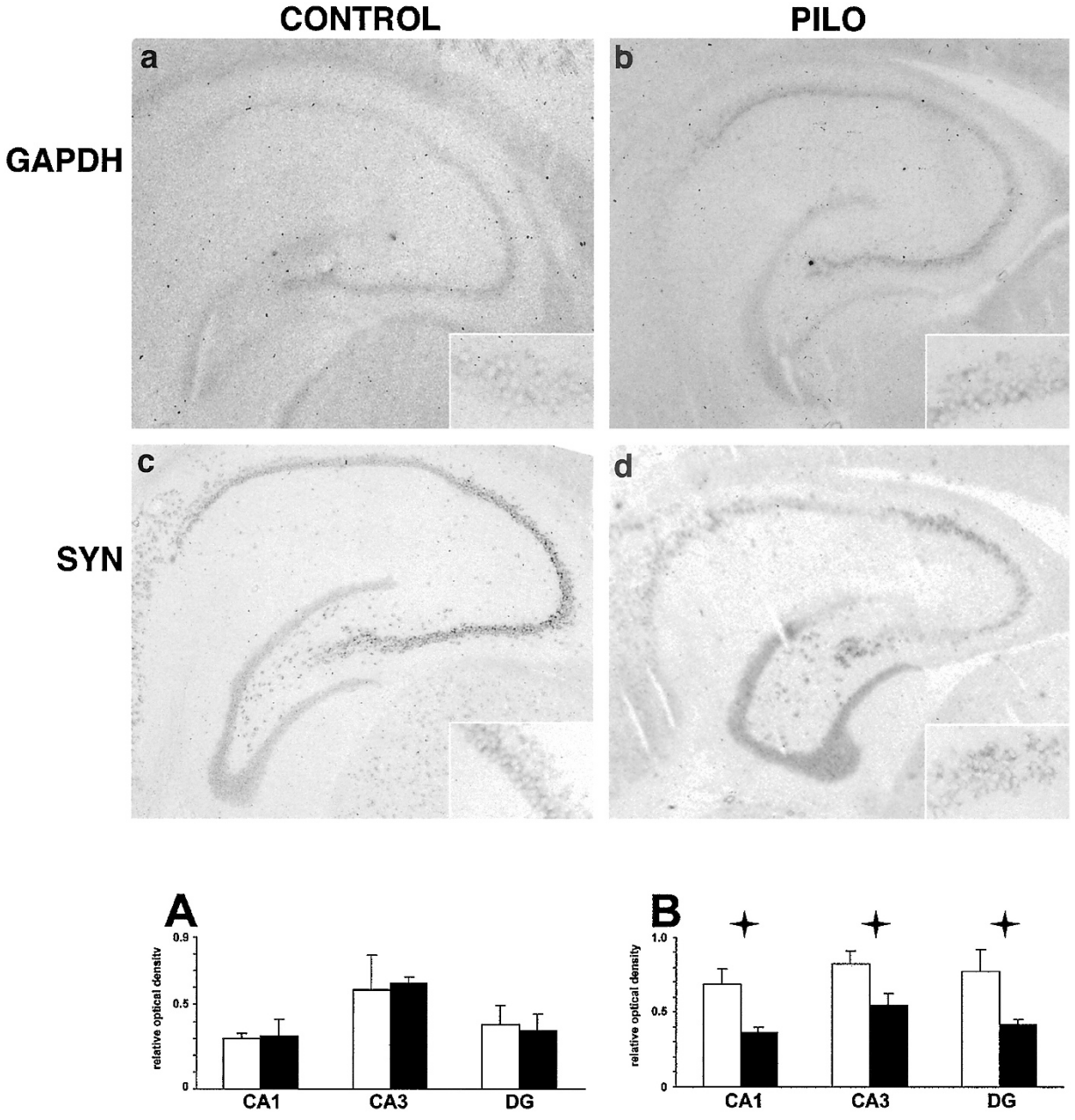

Figure 2.

In situ hybridization analysis of *GAPDH* (a, b) and *synaptophysin* (c, d) expression in hippocampi from control (a and c) vs pilocarpine-treated (b and d) animals (×4). Note the extensive neuronal loss in CA1 and CA3 of pilocarpine-treated animals. Inserts show corresponding CA1 neurons (×10). *Synaptophysin* (A) and *GAPDH* (B) quantitative in situ hybridization data for pilocarpine-treated (white) vs control animals (black) in hippocampal CA1, CA3, and dentate gyrus (DG) areas measured in individual cells. *GAPDH* expression is significantly increased in all areas.

using a dilution series of target gene RT-PCR products (Wang et al, 2000). These standard curves (Fig. 1, A and B; standard dilutions [filled squares]) allowed us to determine mRNA copies of the studied genes in pilocarpine-treated (filled circles) and control rat neurons (empty squares). We observed a significant increase in the expression level of *GAPDH* in pilocarpine-treated versus control animals (1.77-fold; *t* test, $p < 0.005$). In contrast, expression levels for *β-actin* (1.28-fold), *cyclophilin* (1.16-fold), and *HPRT* (1.14-fold) were not significantly different between pilocarpine-treated and control animals. *Synaptophysin* expression was the most stable of the reference genes tested (0.01-fold increase in pilocarpine-treated animals) (Fig. 1C).

To confirm activity-associated regulation of *GAPDH* mRNA, we performed in situ hybridization for *GAPDH* and *synaptophysin* using digoxigenin (DIG)-labeled in vitro transcription products (cRNA) (Hans et al, 1999). Twelve-micrometer cryosections of pilocarpine-treated and control brains ($n = 3$ each) were used for these studies. Hybridization probes for *GAPDH* and *synaptophysin* were generated by PCR using primers for *GAPDH* (5'-CAA GGC TGA GAA TGG GAA G-3';

5'-TTC TGA GTG GCA GTG ATG G-3') and *synaptophysin* (5'-TCT TCC TGC AGA ACA AGT ACC G-3'; 5'-GCC AGG TGC TGG TTG CT-3') with a rat hippocampal cDNA as template and subsequent in vitro transcription. Amplification was carried out in a total reaction volume of 100.0 μl containing 10.0 ng of cDNA, 200 μM dNTPs, 2.5 U *Taq* polymerase, 50 mM KCl, 10 mM TRIS-HCl (pH 8.5), 1.5 mM $MgCl_2$, 0.01% gelatin, and 0.5μM of each primer in an automated thermocycler (Uno Block; Biometra, Göttingen, Germany) for 36 cycles at 94° C for 30 seconds, 58° C for 40 seconds, and 72° C for 40 seconds. Hybridization was performed according to standard procedures (Hans et al, 1999). Control sections were hybridized to sense probes without detecting specific signals. The optical density of hybridization signals was quantified by digital image analysis ($n = 10$ neurons/subfield, MetaView 4.5r5). In situ hybridization showed a significant increase of *GAPDH* mRNA in pilocarpine-treated versus control hippocampi in all subregions despite severe neuronal loss in the CA1 and CA3 subfields of the hippocampus in pilocarpine-treated animals (CA1: 2.12-fold; *t* test, $p < 0.05$; CA3: 1.63-fold; *t* test, $p < 0.05$; dentate gyrus: 2.13-fold; *t* test, $p < 0.05$) (Fig. 2). The signals were mostly observed in neuronal profiles. Synaptophysin mRNA levels in individual neurons were not significantly altered (Fig. 2).

In summary, 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. These changes in expression would introduce a large error into mRNA quantification based on normalization to *GAPDH*. *β-actin*, *cyclophilin*, and *HPRT* were not significantly altered and appear as suitable candidates for reference genes. Surprisingly, when comparing pilocarpine-treated and control animals, the most stable expression was observed for *synaptophysin*. The use of *synaptophysin* as an additional control gene for mRNA-expression studies bears a second advantage: because *synaptophysin* expression is restricted to neurons, mRNA levels will not be affected by an increase in the population of nonneuronal cells also present in brain samples. This approach can be particularly helpful for experiments in tissue slices, which allow one to isolate single neurons. In conclusion, reference genes have to be carefully chosen for mRNA-expression studies in a given experimental paradigm such as pilocarpine-induced seizures. *β-actin*, *cyclophilin*, and *HPRT* represent suitable reference genes, whereas *GAPDH* expression can be significantly modulated in this disease model. The observation of stable *synaptophysin* expression in pilocarpine-exposed hippocampal neurons suggests that, at the mRNA level, the synaptic pool may not be altered in these neurons.

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