Journal of Neuroscience Research 87:34–41 (2009)

Validation of Reference Genes for Normalization of Real-Time Quantitative RT-PCR Data in Traumatic Brain Injury

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Careful validation of reference genes used for the normalization of real-time RT-PCR data is required to obtain accurate results regarding gene expression. We evaluated the stability of seven commonly used reference genes in the cerebral cortex and hippocampus of rats 3 days following traumatic brain injury (TBI). HPRT, SDHA, and GUSB were found to be the most stable reference genes in the cerebral cortex, whereas B2MG, TBP, and GAPDH were the most stable in the hippocampus. The use of three reference genes was determined to be the optimal number for accurate normalization of data. To illustrate this point, when our gene of interest, substance P (SP), was normalized against the three most stable reference genes in both brain areas, we found no significant difference between injured and uninjured rats at the 3-day time point. However, when our SP data were normalized to each reference gene individually, SP mRNA level was highly variable depending on the reference gene chosen. The results of the present study highlight the importance of validating reference genes to be used for real-time RT-PCR analysis. The use of the most stable reference genes presented here will allow more accurate normalization of gene expression data in TBI.

Key words: real-time PCR; substance P; quantitation; rats; neurotrauma

Real-time reverse transcription-polymerase chain reaction (real-time RT-PCR) is currently the most sensitive method for detecting and quantifying mRNA transcripts (Bustin, 2000), and its development has greatly influenced the field of gene expression analysis (Wong and Medrano, 2005a). It has several advantages over traditional end-point RT-PCR, including a wider dynamic range of quantification, higher sensitivity and precision, and a decreased risk of contamination (Klein, 2002; Wilhelm and Pingoud, 2003). The real-time RT-PCR assay incorporates fluorescent molecules to measure the accumulation of PCR products during each cycle, thereby combining amplification and detection (Wong and Medrano, 2005a). The transcript level of the gene of interest is normalized to one or more internal controls, which is necessary to account for differences in quantity and quality of starting material between samples (Radonić et al., 2004; Bustin, 2005). Several strategies exist for the normalization of real-time RT-PCR data, the most popular being the use of a reference gene (Dheda et al., 2005). Such genes should be expressed at a stable level in different tissue types and be unaffected by the experimental condition under investigation (Stürzenbaum and Kille, 2001; Zhu and Altmann, 2005). However, it is well documented that most reference genes are somewhat regulated (Tricarico et al., 2002; Dheda et al., 2004; Radonić et al., 2004; Jain et al., 2006). For example, the transcript level of one of the most commonly used reference genes, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), has been shown to vary considerably under different experimental conditions (Zhong and Simons, 1999; Glare et al., 2002; Aerts et al., 2004; Bas et al., 2004), rendering it unsuitable for normalization in those studies. However, other groups have found the expression of GAPDH to be stable (Ullmannová and Haškovec, 2003; Bäckman et al., 2006; Meldgaard et al., 2006), thus highlighting the importance of evaluating the stability of a chosen reference gene for each new experimental condition (Dheda et al., 2004; Wong and Medrano, 2005a; Meldgaard et al., 2006).

Although it is unlikely that a single reference gene exists that is unaffected by any biological condition in all tissues (Haller et al., 2004), normalization to the geometric mean of the expression of several different reference genes (Vandesompele et al., 2002) is considered to be a reliable and conservative approach (Wong and Medrano, 2005a; Hellemans et al., 2007). Despite the increasing evidence that normalizing real-time RT-PCR data to a single reference gene may be inappropriate, many authors continue to do so without proper validation

Contract grant sponsor: Neurosurgical Research Foundation of Australia; Contract grant sponsor: NHMRC Dora Lush Biomedical Postgraduate Scholarship (to N.L.C.).

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Received 10 February 2008; Revised 12 May 2008; Accepted 12 June 2008

Published online 18 August 2008 in Wiley InterScience (www.interscience.wiley.com). DOI: 10.1002/jnr.21846

(Bustin and Benes, 2005). Indeed, the majority of papers in our area of interest (traumatic brain injury; TBI) describe the use of a single reference gene, such as GAPDH, cyclophilin, β-actin, or 18S ribosomal RNA (Li et al., 2004; Larner et al., 2005; Pascale et al., 2006; Shein et al., 2007; Sifringer et al., 2007; Yao et al., 2007; Brown et al., 2008), usually without including a reference gene validation protocol. Because the expression of these reference genes has been shown to vary across different experimental situations (Zhong and Simons, 1999; Schmittgen and Zakrajsek, 2000), it is uncertain whether these studies have measured a true change in the mRNA of interest or whether data analysis has been influenced by the variability of the reference gene.

Our laboratory has focused on identifying appropriate reference genes for normalizing real-time RT-PCR data related to the mRNA level of the neuropeptide substance P (SP) following TBI. TBI is the leading cause of death and disability in people under 40 years of age (Fleminger and Ponsford, 2005), with motor vehicle accidents accounting for the majority of moderate-severe TBI cases (Khan et al., 2003). Despite the public health burden of TBI, there are no effective treatment options available, such that survivors are often left with debilitating long-term deficits. Delayed secondary injury factors significantly contribute to the morbidity and mortality following TBI (Cormio et al., 1997), and of particular significance is vasogenic edema, which occurs in the setting of blood–brain barrier (BBB) disruption, allowing proteins and water to enter the brain (Heo et al., 2005). Research from our laboratory (Vink et al., 2003; Nimmo et al., 2004) has shown that neurogenic inflammation is central to the genesis of BBB permeability, vasogenic edema, and the functional deficits following diffuse TBI in rats. SP is a potent initiator of neurogenic inflammation, and a rapid increase in SP immunoreactivity has been demonstrated following both TBI (Donkin et al., 2007) and ischaemic brain injury in rats (Turner et al., 2006), the increase in SP being associated with the development of profound cerebral edema. No study, however, has characterized the transcriptional levels of SP following TBI.

In the present study, we have used real-time RT-PCR to characterize the transcript level of SP at 3 days following TBI in rats. However, unlike the case in previous RT-PCR studies of experimental TBI, the identification and validation of suitable reference genes was one of the primary aims of this study. Accordingly, real-time RT-PCR was used to measure transcript levels of seven commonly used reference genes: GAPDH, β-2-microglobulin (β2MG), RNA polymerase II (POL2R), TATA box binding protein (TBP), hypoxanthine guanine phosphoribosyltransferase (HPRT), succinate dehydrogenase complex, subunit A (SDHA), and β-glucuronidase (GUSB) as well as our gene of interest, SP, at 3 days following experimental TBI in rats. We initially applied the relative standard curve method described by Pfaffl (2001) to our data in order to investigate the effect of normalizing to a single reference gene. Next, a reference gene evaluation study was conducted using the freely available geNorm application (Vandesompele et al., 2002) to determine the most stable reference genes from our panel of seven and also the appropriate number of genes required for accurate normalization of our real-time RT-PCR data. Finally, we applied the most stable reference genes to the normalization of SP mRNA level, using the freely available qBase program (Hellemans et al., 2007), which allows the widely used $2^{-\Delta\Delta Ct}$ method of analysis (Livak and Schmittgen, 2001) to be applied with multiple reference genes.

MATERIALS AND METHODS

TBI

All animal protocols were approved by the University of Adelaide Animal Ethics Committee and were conducted according to guidelines established for the use of experimental animals in research by the Australian National Health and Medical Research Council. Adult male Sprague-Dawley rats (n = 12; 350–450 g) were group housed on a 12-hr night-day cycle and provided with a standard diet of rodent pellets and water ad libitum. Animals were randomly assigned into TBI (n = 6) and uninjured (n = 6) groups at the beginning of the experiment. Animals were injured using the impact acceleration model of diffuse TBI as described in detail elsewhere (Marmarou et al., 1994). Briefly, rats were anesthetized with isoflurane, and the skull was exposed by midline incision. A stainless-steel disc (9 mm in diameter and 3 mm in depth) was rigidly fixed with cyanoacrylate adhesive to the skull, placed centrally between the lambda and bregma sutures. Animals were subsequently placed in the prone position on a 10-cm foam bed and subjected to brain injury by dropping a 450-g brass weight a distance of 2 m onto the stainless steel disc. This model of TBI produces widespread axonal injury in the absence of hemorrhage, contusions, and lesions. For comparison with injured animals, sham control animals were surgically prepared but not injured. Animals were maintained at a rectal temperature of 37°C throughout surgery and recovery using a thermostatically controlled heating pad and were returned to their cages after complete recovery. Animals were sacrificed by anaesthetic overdose at 3 days following injury or sham surgery. Brains were rapidly removed and the cerebral cortex and hippocampus regions dissected in total and snap frozen in liquid nitrogen, before being stored at −80°C. Cerebral cortex and hippocampus were chosen for analysis because previous studies from our laboratory (Turner et al., 2004; Donkin et al., 2007) have demonstrated significant neuronal damage in these areas of the brain following TBI.

RNA Extraction

Extraction of total RNA was carried out from TBI and uninjured sham cerebral cortex and hippocampus samples (n = 6 per group, representing left and right cerebral cortex and hippocampus) using Trizol reagent (Invitrogen, Mt. Waverley, Australia) according to the manufacturer's instructions. Briefly, 50 mg tissue was homogenized in 500 μl Trizol and allowed to incubate at room temperature for 5 min before being

TABLE I. Details of Primer Sequences Used in Real-Time RT-PCR Amplification

Symbol	Accession No.	Name	Primer sequences[a] (5′→3′)	Size[b] (bp)	T_A[c]	Reference[d]
SP	NM_012666	Substance P	tggtcagatctctcacaaaagg tgcattgcgcttctttcata	99	60	Novel
GAPDH	NM_017008	Glyceraldehyde-3-phosphate dehydrogenase	tgcaccaccaccctgcttagc ggcatggactgtggtcatgag	87	57	Li et al., 2003
β2MG	NM_012512	β-2-Microglobulin	acatcctggctcacactgaa atgtctcggtcccaggtg	109	60	Novel
POL2R	XM_001079162	RNA polymerase II	tttgaggaaacggtggatgt tggcccagcataatattctca	92	56	Novel
HPRT	NM_012583.2	Hypoxanthine guanine phosphoribosyltransferase	ttgttggatatgcccttgact ccgctgtctttttaggctttg	105	60	van Wijngaarden et al., 2007
GUSB	NM_017015	β-Glucuronidase	tccttccatgtatcccaagg tggtaggggtggtgtacagg	104	60	Novel
TBP	NM_001004198	TATA Box Binding Protein	cagccttccaccttatgctc tgctgctgtctttgttgctc	165	60	Pohjanvirta et al., 2006
SDHA	AB072907	Succinate dehydrogenase complex, subunit A	agacgtttgacaggggaatg tcatcaatccgcaccttgta	160	60	Pohjanvirta et al., 2006

[a]Forward primer sequence on upper line, reverse sequence on lower line.
[b]Amplicon lengths in base pairs.
[c]T_A indicates optimum annealing temperature (°C).
[d]Novel indicates that primers were designed by our laboratory using Primer3Plus software.

mixed with 100 μl chloroform and centrifuged for 15 min at 13,200 rpm. The aqueous phase was mixed with 250 μl isopropanol, incubated at room temperature for 10 min and centrifuged for 20 min at 12,000 rpm. After removal of the isopropanol, the precipitate containing the RNA was mixed with 500 μl 70% ethanol and centrifuged for 10 min at 13,200 rpm. The precipitate was allowed to air dry before being resuspended in nuclease-free water (Qiagen, Doncaster, Australia). RNA samples were treated with the Qiagen RNase-free DNase set to remove contaminating genomic DNA, then purified and concentrated using the RNeasy MinElute clean-up kit (Qiagen) according to the manufacturer's instructions. Total RNA was quantified using the Nanophotometer (Implen, Australia) by measuring absorbance at 260, 280, and 320 nm. RNA integrity was assessed by using the Agilent Bioanalyzer RNA 6000 Nano Chip (Series II) kit.

Reverse Transcription

Complementary DNA was synthesized by using the SuperScript III Reverse Transcriptase kit (Invitrogen). Two micrograms of total RNA was added to 250 ng random hexamers (Geneworks, Adelaide, Australia), 1 mM each dNTP (Bioline, Sydney, Australia), and nuclease-free water to 13 μl. Reactions were heated to 65°C for 5 min, then immediately placed on ice for 1 min. To each tube, 4.75 μl 5× first strand buffer, 1 μl RNase OUT (Invitrogen), 0.02 M dithiothreitol, and 200 units SuperScript III reverse transcriptase were added. Reactions containing nuclease-free water in place of enzyme served as negative controls. Reactions were incubated at 25°C for 5 min, 55°C for 60 min, and 70°C for 15 min. cDNA was diluted to 10 ng/μl with nuclease-free water and stored at −20°C.

Primer Design

Details of genes studied, including forward and reverse primer sequences, amplicon sizes, and annealing temperatures are detailed in Table I. Primers were designed in Primer3Plus software (Untergasser et al., 2007), except where noted. To exclude amplification of genomic DNA, primers were designed to span an exon–intron boundary wherever possible. Primer complementarity was evaluated in NetPrimer (http://www.premierbiosoft.com/netprimer/netprlaunch/netprlaunch.html). Primers were assessed for specificity in PUNS software (Boutros and Okey, 2004) to ensure production of a single amplicon specific to the mRNA transcript.

Real-Time PCR

Optimal reaction conditions for real-time RT-PCR were obtained with 10 μl 2× Platinum SYBR Green SuperMix-UDG (Invitrogen), 300 nM forward and reverse primers (400 nM for GAPDH and POL2R), 2 μl cDNA, and nuclease-free water in a total volume of 20 μl. For POL2R, 100 nM $MgCl_2$ (Invitrogen) was added. Amplification was carried out in a Corbett Rotor-Gene 3000 with an initial UDG incubation of 50°C for 2 min, initial denaturation of 95°C for 2 min, followed by 40 cycles of: 95°C for 15 sec denaturation, x°C for 15 sec annealing, and 72°C for 15 sec extension (x°C refers to the annealing temperatures for individual primer pairs, as listed in Table I). Fluorescence data were collected during the extension step of each cycle. Specificity of amplicons was verified by melting curve analysis after 40 cycles (72–95°C) and 2% agarose gel electrophoresis (data not shown). Unknown cDNA samples were run in triplicate, with a set of standards (in triplicate) also included in each run, comprising fivefold serial dilutions made from aliquots of pooled cDNA, derived from an RNA pool of all samples. Serial dilutions comprised the following input amounts of cDNA: 100, 20, 4, and 0.8 ng. Negative controls containing water instead of cDNA were present in all runs.

Data Analysis

After PCR amplification, the cycle threshold (Ct) and raw values of input RNA were calculated from the standard

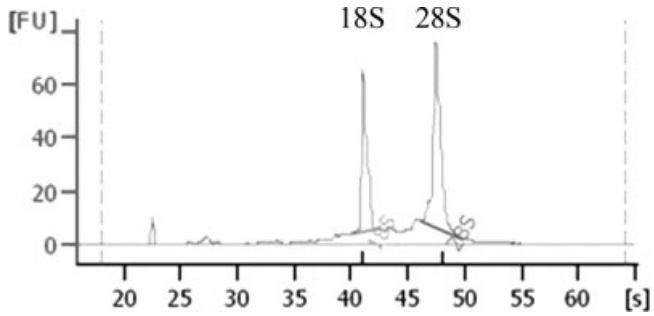

Fig. 1. Representative electropherogram from the Agilent Bioanalyzer Expert 2100 software, showing intact RNA sample with an RIN of 9.0. Labelled 18S and 28S rRNA peaks are clearly visible. FU, fluorescence units; s, seconds.

curve using the Corbett Rotor-Gene 6 software. The Ct of an individual sample reflects the cycle at which detectable numbers of PCR products have accumulated above background fluorescence (Wilhelm and Pingoud, 2003). *Raw values* refer to uncorrected data that have not yet been normalized to the reference genes. The relative standard curve method (Pfaffl, 2001) was applied to the raw quantities of input RNA to calculate SP mRNA level relative to each of the seven reference genes individually. The raw values of RNA obtained for the seven reference genes were also entered into the geNorm v3.5 software (Vandesompele et al., 2002) and subjected to expression stability analysis by pairwise correlations among all samples. Subsequently, the most stable reference genes were applied to the normalization of SP transcript level, using the qBase v1.3.5 program (Hellemans et al., 2007). Statistical analysis was by unpaired Student's *t*-test.

RESULTS

Assessment of RNA Concentration and Integrity

The concentration of extracted total RNA was quantified spectrophotometrically by measurement of the absorbance at 260, 280, and 320 nm. A260:A280 ratios of between 1.9 and 2.2 and A260:A230 ratios of between 1.8 and 2.2 were obtained for all samples, indicative of relatively pure RNA. RNA integrity was assessed by using automated microcapillary electrophoresis in an Agilent Bioanalyzer. An RNA Integrity Number (RIN; for review see Schroeder et al., 2006) was assigned to each sample by the Agilent Bioanalyzer Expert 2100 software. All RINs obtained were in the range of 8.0–9.3, indicating high-quality RNA with minimal degradation (Fig. 1).

Real-Time RT-PCR

Assay optimization. Primer pairs for all genes were tested using real-time RT-PCR on the standard cDNA pool. A primer concentration optimization assay was carried out to determine the optimal concentrations for each primer pair. Melting curve analysis was consistent with a single reaction product for each gene, and product size was confirmed by 2% agarose gel electrophoresis. No-reverse transcriptase controls were included to test for spurious genomic DNA amplification. After validation of primer specificity, real-time RT-PCR was carried out for unknown TBI and sham cDNA samples and serial dilutions from the cDNA pool. Each dilution step of the cDNA standards (100, 20, 4, and 0.8 ng) could be amplified. Reaction efficiencies were calculated automatically by the Corbett Rotor-Gene 6 software according to the equation: Reaction efficiency = $(10^{[-1/\text{slope}]} - 1) \times 100$. All runs exhibited efficiencies between 95% and 105%. Minimum R^2 values of 0.98 were accepted for each run.

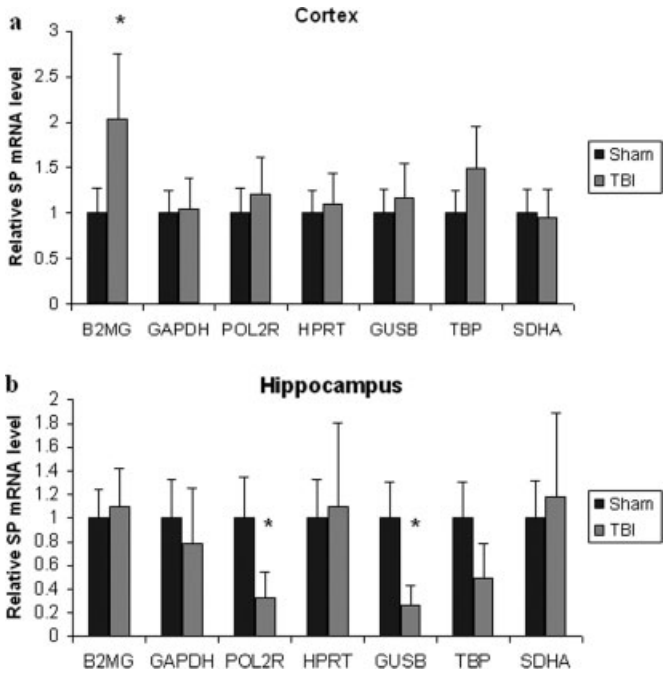

Fig. 2. Relative SP mRNA level in the cerebral cortex (**a**) and hippocampus (**b**) of TBI (n = 6) and uninjured rats (n = 6), normalized to individual reference genes. Bars represent mean SP mRNA values ± SEM. Sham expression is set to 1 to facilitate determination of -fold changes in mRNA level. $\star P < 0.05$.

SP mRNA level normalized to individual reference genes. The relative standard curve method (Pfaffl, 2001) was used to calculate SP mRNA level in the cerebral cortex and hippocampus, relative to each of our seven reference genes individually. In the cerebral cortex, when SP was normalized to β2MG only, a significant ($P < 0.05$) twofold increase in mean SP mRNA level was observed in the TBI group compared with uninjured shams (Fig. 2a). When each of the other reference genes was used individually for normalization, there was no significant difference ($P > 0.05$) in mean SP mRNA level between TBI and uninjured rats.

In the hippocampus, when GUSB and POL2R were used as single reference genes, a significant ($P < 0.05$) decrease in SP mRNA level was observed, such

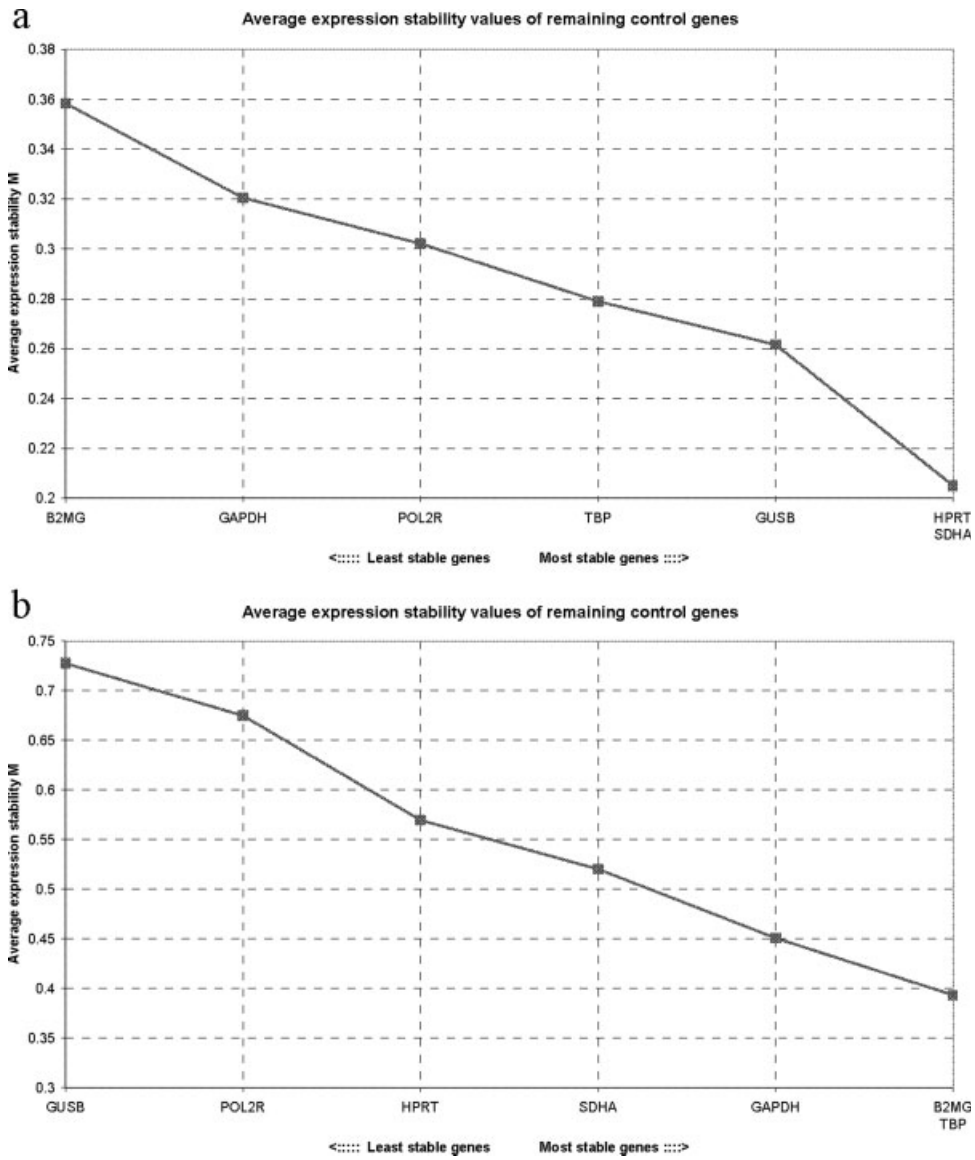

Fig. 3. Reference genes ranked in order of stability, as determined by geNorm in cerebral cortex (**a**) and hippocampus (**b**). Genes with lower M values have the most stable expression.

that mean SP levels in the TBI group were 25% and 33% of the sham (uninjured) group, respectively (Fig. 2b). When the remaining five reference genes were used individually for normalization of our SP data, there was no significant ($P > 0.05$) difference in mean SP mRNA level between TBI and uninjured animals.

Given the discrepancy in these results and the large variation between samples, particularly in the hippocampus, we proceeded with a reference gene evaluation study to determine the most stable reference genes in both regions of the brain.

Reference gene stability. For each of the seven candidate reference genes, raw expression values of each sample relative to the standard curve were entered into the geNorm v3.5 application. For each gene under investigation, geNorm calculates a gene stability measure, M, based on geometric averaging of multiple control genes and average pairwise variation of a particular gene with all other control genes in a given set of samples. The lower the value of M, the more stable the expression of the candidate reference gene (Vandesompele et al., 2002). The most stable reference genes in the present study were HPRT and SDHA in the cerebral cortex and β2MG and TBP in the hippocampus (Fig. 3). Pairwise variation analysis by geNorm recommended three reference genes as the optimal number for accurate normalization in both brain regions studied (data not shown). Therefore, the final reference genes chosen

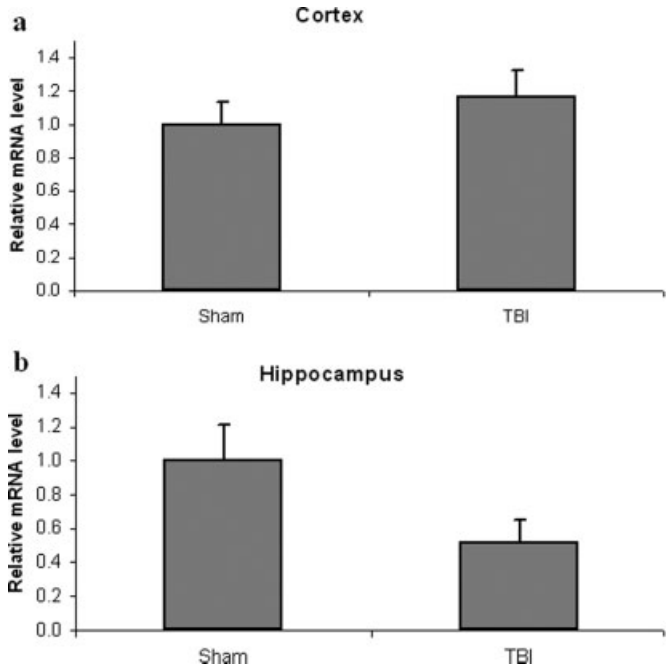

Fig. 4. Relative SP mRNA level in sham (n = 6) and TBI (n = 6) animals, normalized to the three most stable reference genes in cerebral cortex (**a**; HPRT, SDHA, and GUSB) and hippocampus (**b**; B2MG, TBP, and GAPDH). Bars represent mean SP mRNA values ± SEM.

were HPRT, SDHA, and GUSB for cerebral cortex and β2MG, TBP, and GAPDH for hippocampus.

SP mRNA level using multiple reference genes. The qBase program was used to calculate the normalized mRNA level of SP relative to the three most stable reference genes determined by geNorm. qBase utilizes a modified version of the classic $2^{-\Delta\Delta Ct}$ method of relative expression analysis (Livak and Schmittgen, 2001) that takes into account multiple reference genes and gene-specific amplification efficiencies (Hellemans et al., 2007). In both the cerebral cortex and the hippocampus groups, there was no significant difference in mean SP transcript level of SP between TBI and sham animals ($P > 0.05$). However, there was a trend ($P = 0.08$) toward SP mRNA level decreasing in the hippocampus in the injured group (Fig. 4).

DISCUSSION

Real-time RT-PCR is becoming the method of choice for high-throughput gene expression analysis because of its wide dynamic range of quantification, high sensitivity and precision, and decreased risk of contamination compared with traditional end-point RT-PCR (Klein, 2002; Wilhelm and Pingoud, 2003). It requires an appropriate normalization strategy to control for error, the most common being the use of one or more endogenous reference genes (Nolan et al., 2006). A reference gene should be expressed at a stable level regardless of the experimental context; however, several studies have demonstrated that the expression of reference genes can vary considerably (Tricarico et al., 2002; Dheda et al., 2004; Radonić et al., 2004; Jain et al., 2006). Therefore, we initially considered using an alternative method of normalization, such as an exogenous RNA standard. Exogenous standards represent an accurate method of normalization because a known amount of standard can be incorporated into the RNA extraction process, which will then be affected by the same experimental error as the RNA of interest (Huggett et al., 2005). Exogenous standards can either be in vitro transcribed RNA that is homologous to the RNA of interest apart from a small insertion, deletion, or mutation or an artificial molecule with no homology to the RNA of interest (Freeman et al., 1999). Several groups have reported reliable normalization with exogenous reference standards (Ke et al., 2000; Smith et al., 2003; Moriya et al., 2006). However, exogenous RNA standards can be difficult to produce and are subject to degradation. Also, they do not control for differences in quality of RNA template, meaning that an internal control, such as a reference gene, would still be required (Wong and Medrano, 2005b). Therefore, with these considerations in mind, we chose to use multiple reference genes to normalize our data.

Careful evaluation of reference gene stability is necessary to generate reliable and biologically meaningful results with real-time RT-PCR. The primary aim of this study was to validate suitable reference genes for normalizing real-time RT-PCR data in experimental TBI. Real-time RT-PCR was performed to measure transcript levels of seven reference genes and one gene of interest, SP, in TBI and sham (uninjured) rats, at 3 days postinjury. The time point of 3 days was chosen because our previous immunohistochemistry studies have shown increased SP immunoreactivity at 3 days following TBI in both the cerebral cortex and the hippocampus (Donkin et al., 2007). Initially, data regarding SP transcript level were normalized to individual reference genes using the relative standard curve method of analysis described by Pfaffl (2001). When SP cerebral cortex data were normalized to β2MG only, a significant ($P < 0.05$) twofold increase in mean SP transcript level was observed in the TBI group compared with uninjured animals. When the same SP data were normalized to the other six reference genes individually, there was no significant difference ($P > 0.05$) in mean SP transcript level between TBI and uninjured rats, and, furthermore, there was a large variation in relative transcript level between samples. Similarly, in the hippocampus, when SP data were normalized to either GUSB or POL2R only, a significant ($P < 0.05$) decrease in mean SP transcript level was observed. However, when normalized individually to the other five reference genes, there was no significant difference ($P > 0.05$) in mean SP transcript level between TBI and uninjured rats. In both regions of the brain, normalizing to a single reference gene produced a considerable variation of relative SP transcript level between samples, which is clearly demonstrated by the large error bars in Figure 2. However, when geNorm was used to assess the stability of our seven reference

genes and to select the three most stable genes for normalizsing our SP data, far more consistent results were obtained (as can be seen in Fig. 4). Interestingly, when we assessed the stability of the seven reference genes using geNorm, β2MG (which generated a statistically significant result when normalizing cerebral cortex SP data) was found to have the least stable expression in the cerebral cortex. Likewise, GUSB and POL2R, which produced statistically significant results when normalizing hippocampus SP data, were the least stable reference genes in the hippocampus. Such findings highlight the importance of careful selection and validation of reference genes to be used for real-time RT-PCR normalization and demonstrate that normalization to the geometric mean of multiple reference genes (Vandesompele et al., 2002) generates more reproducible and consistent results than using a single reference gene.

We found the freely available geNorm and qBase applications to be convenient tools for analyzing our real-time RT-PCR data. geNorm quickly determined the stability of each of our reference genes and provided the optimal number that should be applied for accurate normalization. It is interesting that the three most stable reference genes in the cerebral cortex (HPRT, SDHA, and GUSB) were different from those in the hippocampus (β2MG, TBP, and GAPDH). The animal model of TBI used in this study (Marmarou et al., 1994) produces diffuse axonal injury with widespread edema and neuronal damage throughout the cortex and hippocampus but does not produce localized lesions, such as contusions or hemorrhage. At this point, we do not know why there is a difference in the mRNA level of our reference genes between the cortex and the hippocampus. It is possible that the differences in transcript levels of the reference genes can be attributed to the local effects of necrosis, degeneration, inflammation, or edema in the different areas of the brain, and this warrants further investigation. This finding emphasizes the importance of evaluating reference gene stability in different tissue types and, indeed, different areas of the brain, for each experimental condition under investigation.

The qBase program calculated the relative transcript level of SP normalized to the three most stable reference genes determined by geNorm. There was no significant difference ($P > 0.05$) in mean SP transcript level between TBI and sham (uninjured) animals, in either the cerebral cortex or the hippocampus. However, we observed a trend toward a decrease in SP transcript level in the hippocampus ($P = 0.08$), which contrasts with our immunohistochemistry studies in which we found an increase in SP immunoreactivity at 3 days following TBI (Donkin et al., 2007). In the same study, we also found an increase in SP immunoreactivity at 5 and 24 hr postinjury. Future studies will characterize the time course of SP transcript level ranging from 5 hr to 1 week following injury, with a reference gene validation study similar to the one described here incorporated to determine which reference genes are the most stable in both the acute and the prolonged phases following TBI.

In conclusion, the results of the present study demonstrate the importance of identifying and validating suitable reference genes for real-time RT-PCR normalization and are consistent with other studies in which the choice of reference gene has profoundly influenced the biological interpretation of real-time RT-PCR data (Aerts et al., 2004; Dheda et al., 2005). We have evaluated the stability of seven reference genes in experimental TBI and have identified HPRT, SDHA, and GUSB as the most stable reference genes in the cerebral cortex and β2MG, TBP, and GAPDH in the hippocampus. The use of these reference genes will allow more accurate normalization of real-time RT-PCR data at a 3-day time point following experimental TBI.

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