

Selection of housekeeping genes for normalization of RT-PCR in hypoxic neural stem cells of rat in vitro

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Abstract Gene expression analysis under various conditions using real-time reverse transcription polymerase chain reaction (RT-PCR) needs reliable control genes. Housekeeping genes are commonly used as the control. However, no validated housekeeping gene is available for study of hypoxic neural stem cell culture. To choose appropriate internal control genes, the expression of eight commonly used housekeeping genes was examined in rat neural stem cell model to find one or more stably expressed genes under hypoxic/ischemic conditions. Two genes, HPRT and RPL13A were identified as the most confidential housekeeping genes in this research by geNorm and NormFinder softwares. As a groundwork, the most stable housekeeping genes for neural stem cells under hypoxic/ischemic conditions are initially investigated and validated in this experiment, which might provide a better understanding for the gene expression study in ischemic and necrotic neural stem cell cultures or in ischemic diseases of the central nervous system (CNS).

Keywords Housekeeping gene · Neural stem cells · Hypoxic condition · RT-PCR

Introduction

Neural stem cells are generally considered as highly hypoxia-sensitive among all kinds of cells and play the most important role in nervous system repair following hypoxic/ischemic injury. In recent years, the effects of hypoxia/ischemic on gene expression in nerve cells especially in neural stem cells have been widely investigated. RT-PCR is an invaluable tool for the measurement of target genes in neural stem cells under the hypoxic condition [10, 12, 18]. Measuring an internal reference or housekeeping gene is by far the most commonly used method and also the most reliable one. The sensitivity and accuracy of RT-PCR data are dependent on the reliable reference within each sample to normalize for experiment variations.

Housekeeping genes are used under the assumption that their expression is invariable under any experimental conditions. An ideal reference gene for real-time RT-PCR should be expressed consistently and at the comparable level in all the samples under investigation. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH), hypoxanthine guanine phosphoribosyl transferase (HPRT), cyclophilin A (PPIA), ribosomal protein L13A (RPL13A) and β -actin (Actb) are commonly used housekeeping genes as internal controls in quantitative mRNA analysis for gene expression study of neural stem cells under hypoxia [1, 8]. However, none of them always manifests stable expression under all of these experimental conditions [4, 9, 14, 19, 23, 29]. Unsuitable reference genes may weaken the detection sensitivity of the target genes and even result in inaccurate results [10, 12, 18]. Few validated housekeeping genes have been reported for

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the relative quantification of the mRNA expression profile activated in ischemic and necrotic conditions of neural stem cell models. Brain creatine kinase (Ckb) was found to have the highest expression in brain [27]. Eukaryotic translation elongation factor 1 alpha 1 (Eef1 α 1) was adopted as a reliable housekeeping gene under the influence of factors such as warm ischemia and time at room temperature [2]. Porphobilinogen deaminase (PBG-D) has been hypothesized as a suitable control gene in rat model of hyperglycemic focal cerebral ischemia [5] and for gene expression detection in human placental tissue and leukocytes when subjected to severe tissue hypoxia in neonates with birth asphyxia [26].

The aim of this study is to develop a set of reference genes that can be used for normalization of RT-PCR gene expression data in ischemic and necrotic neural stem cell cultures. The validity of eight reference genes was investigated by RT-PCR under hypoxic/ischemic conditions compared with normoxic condition in primary cultured neural stem cells of rat.

Materials and methods

Rat neural stem cell culture

A pregnant rat with E15 fetus was anaesthetized with chloral hydrate (3.5 ml/kg). Fetuses were then surgically removed and the telencephalon was dissected under sterile condition with the aid of a stereo dissecting microscope. Meninges were removed from the brains and only cortical tissue was used for culture. After removal of the meninges, the freshly prepared fetal cortex was incubated in dissection buffer [0.01% trypsin,; 200 M EDTA, 0.6% glucose, 1 mM MgCl₂ in PBS (all from Sigma, St Louis, MO, USA)] at 37°C for 10 min. Trypsin was quenched with 0.2 mg/ml soybean trypsin inhibitor (Roche Diagnostics, Indianapolis, IN, USA), and then the tissues were mechanically dissociated with a fire-polished Pasteur pipette into single-cell suspensions. The single cell suspension was obtained using filter membrane with three hundred screen meshes. The cell suspension was cultured in DMEM/F12 (1:1) medium (GIBCO/BRL) with 1% B-27 supplement (Invitrogen), 1% N2 supplement (Invitrogen), 10 ng/ml EGF (Invitrogen) and 10 ng/ml bFGF (Invitrogen). For suspension culture, 1 × 10⁶ cells in 4 ml of the same medium were inoculated into a cell culture flask. The cell cultures were maintained in a humidified incubator in 5% CO₂ at 37°C.

Hypoxia exposure

After three generations, the rat neural stem cells were incubated under 0.3% O₂/94.7% N₂/5% CO₂ in Bugbox-M

workstation (Ruskin) condition for 6 h and then put back to the normoxic incubator [7]. Control cultures were incubated in normal (20% O₂/75% N₂/5% CO₂) O₂ conditions separately during this experiment. Rat neural stem cell samples were collected from the hypoxic groups and controls at 0, 3, 6, 12 and 24 h after reperfusion (*n* = 3 in each group). Then, equal numbers of cells were used for RNA isolation.

Isolation of total RNA and reverse transcription

The normoxic or hypoxic rat neural stem cells were washed with ice-cold PBS. Total RNA was prepared using TRIzol Reagent (Invitrogen, CA) and was then treated with DNase I (Sigma) to eliminate residual genomic DNA. The quality and quantity of total RNA was assessed by the ratio of the optical densities at 260 and 280 nm by CE 2301 Low Cost DNA/RNA GeneQuest Analyser (Cecil). The integrity of total RNA was checked by 1% agarose gel electrophoresis. Pure total RNA (1 mg) was subjected to cDNA synthesis using First Strand cDNA Synthesis Kit (Fermentas). Both Oligo (dT) and Random primers were used in a reverse-transcription (RT) reaction. All samples were run as triplicates.

Selection of primers

Primers were designed with Oligo5.0 (Molecular Biology Insights, Cascade, CO) and synthesized by TaKaRa. All primers were designed to be as close as possible to the 3' end of the RNA sequence, and we attempted to locate them on different exons so as to avoid DNA contamination. The primer sequences are shown in Table 1. This was used to assess expression, variability and reproducibility of potential housekeeping genes.

RT-PCR

The quality and quantity of synthesized cDNAs were assessed by the ratio of the optical densities at 260 and 280 nm by CE 2301 Low Cost DNA/RNA GeneQuest Analyser (Cecil). The reactions contained 1× Sybrgreen reaction, each primer at 100 nM, and 2 μg of cDNA template in a 20 μl reaction volume. Amplification was performed by an initial denaturation step at 95°C for 60 s, followed by 40 cycles of denaturation at 95°C for 15 s, annealing at 60°C for 15 s, and then extension at 72°C for 45 s. The amplification specificity was confirmed by melting-curve analysis of the PCR products. RT-PCR was performed by using iQ5 (Bio-Rad). Non-template and non-RT control were used and all samples were run as triplicates.

Table 1 Candidate reference genes

Gene symbol and accession number	mRNA gene name	Primer sequence	Product length
Ckb NM_012529	Brain creatine kinase	F 5'-CTGGCAGGTACTATGCGCTCAA-3' R 5'-TTGATCCACACCAGGAACGTCTTA-3'	181
HPRT NM_012583	Hypoxanthine guanine phosphoribosyl transferase	F 5'-TCCTCATGGACTGATTATGGACA-3' R 5'-TAATCCAGCAGGTCAGCAAAGA-3'	132
GAPDH NM_017008	Glyceraldehyde-3-phosphate dehydrogenase	F 5'-GGC ACAGTCAAGGCTGAGAATG-3' R 5'-ATGGTGGTGAAGACGCCAGTA-3'	143
Actb NM_031144	β -actin	F 5'-GGAGATTACTGCCCTGGCTCCTA-3' R 5'-GACTCAICGTAICTCTGCTTGCTG-3'	150
RPL13A NMJ73340	Ribosomal protein L13A	F 5'-TCTCCGAAAGCGGATGAACAC-3' R 5'-CAACACCTTGAGGCGTTCCA-3'	145
Eef1cl NM_175838	Eukaryotic translation elongation factor 1 alpha 1	F 5'-ATCAAGAACATGATTACGGGCACA-3' R 5'-GGTCTGCCCGTCTTGAGATA-3'	105
PBG-D NM_013168	Porphobilinogen deaminase	F 5'-AGATGGCTCAGATAGCATGCAAGA-3' R 5'-TTCCGGGCAGTGATTCCAA-3'	111
PHA NM017101	Cyclophilin A	F 5'-GGCA AATGCTGGACC AAACAC-3' R 5'-AAACGCTCCATGGCTTCCAC-3'	135

Statistical analysis

All the data were expressed as mean \pm standard error of the mean (SEM) from three independent experiments with triple RT-PCR reactions for each housekeeping gene. One-way analysis of variance (ANOVA) test was applied to analyze significant differences between normoxic and hypoxic/ischemic conditions for each housekeeping gene. Differences between the expression of each housekeeping gene at each time point after reperfusion and that of control group were assessed by the Dunnett's test and were considered significant at $P < 0.05$. All the calculations were performed using GraphPad Prism (version 5.0).

Determination of reference gene expression stability

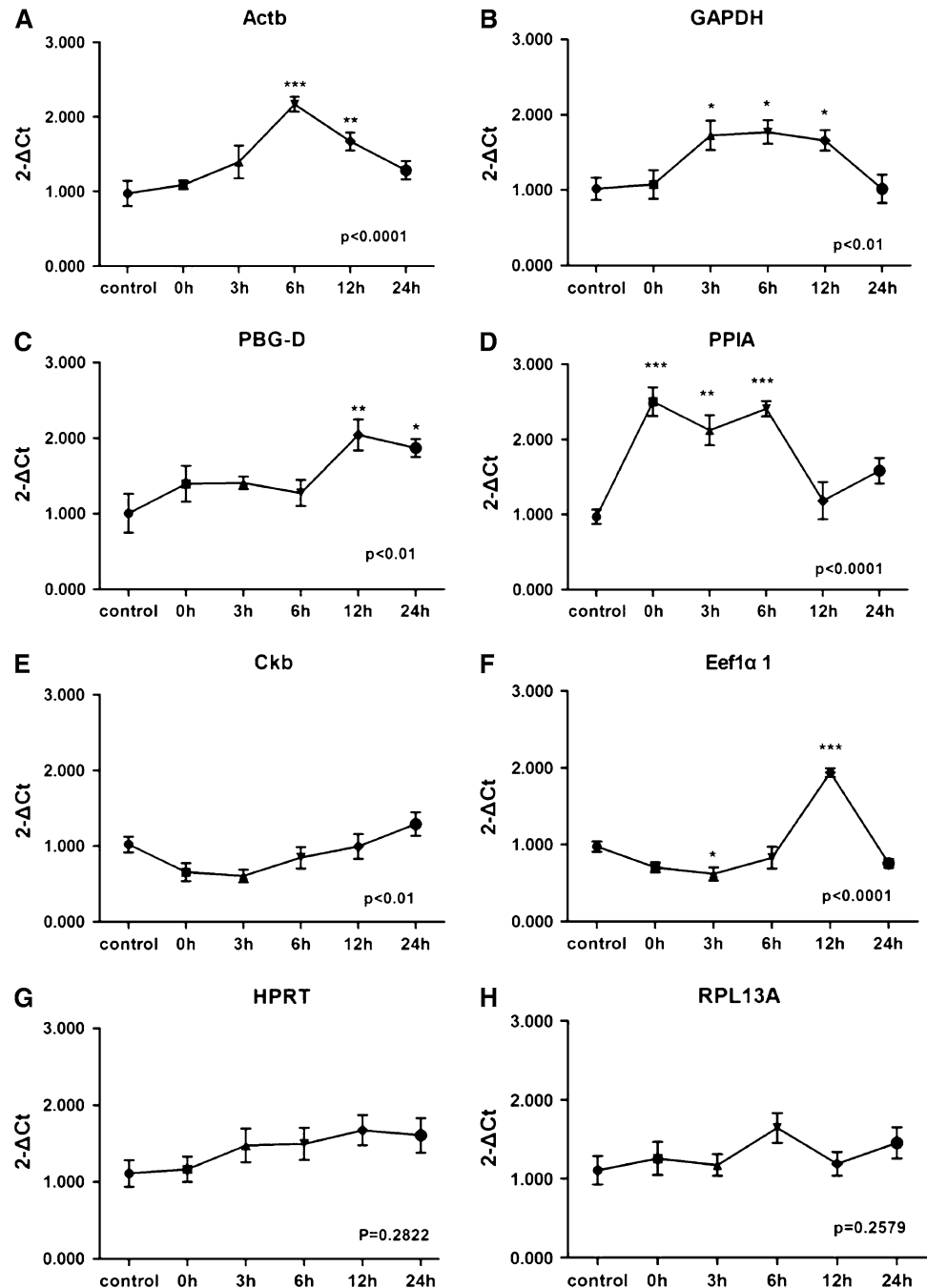
To rank the expression stability of housekeeping genes by the $2^{-\Delta C_T}$ method and ANOVA + post-hoc analysis in vitro model, we used two public available software tools, geNorm [28] and NormFinder [3], to analyze the stability of gene expression. geNorm calculates the gene stability value M, which is the average of a particular gene mutation paired with all other candidates for reference genes. A lower value of M reflects a more stable reference gene. NormFinder estimates the normalization of gene expression changes in the candidates and the overall change between the groups of the sample set used in model-based approach.

Results

The gene expressions of the eight reference genes from the rat neural stem cells exposed under hypoxic condition for 6 h or under normoxic condition, and those collected at 0, 3, 6, 12 and 24 h after reperfusion are shown in Fig. 1.

The Actb mRNA increased significantly at 6 h ($P < 0.0001$) and 12 h ($P < 0.01$) during reperfusion (Fig. 1a). The expression of GAPDH was found increased significantly at 3, 6 and 12 h during reperfusion ($P < 0.05$) whereas no significant changes were observed at other points (Fig. 1b). PBG-D also showed significant differences in the mRNA expression among groups ($P < 0.01$). The expression was stable at the first three analysis time points but increased significantly at 12 h ($P < 0.01$) and 24 h ($P < 0.05$) (Fig. 1c). There was difference in the expression of PPIA among time groups ($P < 0.0001$), expression levels increasing at 0, 3 and 6 h ($P < 0.0001$ in three cases) in comparison with the control group (Fig. 1d). Notable fluctuation also existed in the mRNA expression of Ckb ($P < 0.01$) (Fig. 1e). Changes in Eef1 α 1 mRNA levels were found to be significant among the time groups ($P < 0.0001$). The expression of Eef1 α 1 decreased at 3 h ($P < 0.05$) and increased at 12 h ($P < 0.0001$) in comparison with the control group (Fig. 1f). The expression of HPRT, in comparison to the control group, presented no remarkable fluctuation in all the reference gene groups as a whole ($P = 0.2822$) (Fig. 1g). Analysis of the same samples with a specific gene expression assay for RPL13A did not reveal any remarkable differences in expressions at

Fig. 1 Effects of hypoxia on housekeeping gene expression in the rat neural stem cells. **a** Actb, **b** GAPDH, **c** HPRT, **d** PPIA, **e** Ckb, **f** Eef1 α 1, **g** PBG-D, and **h** RPL13A. Fold change in gene expression analysed by the 2- Δ CT (see “Materials and methods” section for details). Data are mean \pm S.E.M., $n = 3$: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.0001$ versus controls



different time points ($P = 0.2579$) (Fig. 1h). HPRT and RPL13A were found to show the least expression variation. The results of each housekeeping gene expression at different time points in reperfusion after ischemia compared with non-ischemic control group are summarized in Table 2.

In this study, the average expression stability M sequence from lower to higher is HPRT, RPL13A, PBG-D, GAPDH, Eef1 α 1, Ckb, Actb and PPIA by geNorm (Fig. 2a). As the M value is smaller when the stability of gene expression is higher, the greater the M value, the lower the stability of gene

expression. Therefore, the selected eight housekeeping genes from higher to lower stability are HPRT-RPL13A > PBG-D > GAPDH > Eef1 α 1 > Ckb > Actb > PPIA. In geNorm program, the value 0.15 is to determine the appropriate number of housekeeping genes. This test $V2/3 = 0.053$ (< 0.15) (Fig. 2b), so the optimal number of genes is 2, respectively, the two former as HPRT and RPL13A.

After NormFinder calculation, the stability values of HPRT, RPL13A, PBG-D, GAPDH, Eef1 α 1, Ckb, Actb and PPIA were 0.004, 0.004, 0.006, 0.006, 0.016, 0.021, 0.039, 0.064. NormFinder ensured HPRT and RPL13A as

Table 2 Fold change of each housekeeping gene expression at 0, 3, 6, 12 and 24 h time points in reperfusion after ischemia compared with non-ischemic control group

Group	Actb	GAPDH	PBG-D	PPIA
Control	0.97 ± 0.17	1.02 ± 0.15	1.01 ± 0.26	0.97 ± 0.10
0 h	1.09 ± 0.06	1.08 ± 0.19	1.40 ± 0.24	2.50 ± 0.19**
3 h	1.40 ± 0.22	1.74 ± 0.19*	1.41 ± 0.08	2.12 ± 0.20**
6 h	2.17 ± 0.10**	1.77 ± 0.16*	1.28 ± 0.17	2.41 ± 0.10**
12 h	1.67 ± 0.12*	1.66 ± 0.14*	2.04 ± 0.20**	1.19 ± 0.25
24 h	1.29 ± 0.12	1.02 ± 0.19	1.87 ± 0.12*	1.58 ± 0.17
Group	Ckb	Eef1α1	HPRT	RPL13A
Control	1.02 ± 0.10	0.98 ± 0.07	1.11 ± 0.15	1.11 ± 0.16
0 h	0.66 ± 0.12	0.70 ± 0.07	1.17 ± 0.14	1.26 ± 0.19
3 h	0.61 ± 0.08	0.62 ± 0.08*	1.48 ± 0.20	1.17 ± 0.12
6 h	0.85 ± 0.14	0.83 ± 0.14	1.50 ± 0.19	1.64 ± 0.17
12 h	1.00 ± 0.16	1.94 ± 0.06	1.67 ± 0.18	1.19 ± 0.13
24 h	1.29 ± 0.16	0.76 ± 0.06*	1.61 ± 0.20	1.45 ± 0.18

Data are means ± SEM, $n = 3$

* $P < 0.05$, ** $P < 0.01$ versus controls

the most stable gene with a stability value of 0.004 and, as with geNorm, HPRT and RPL13A were also chosen as the most suitable gene.

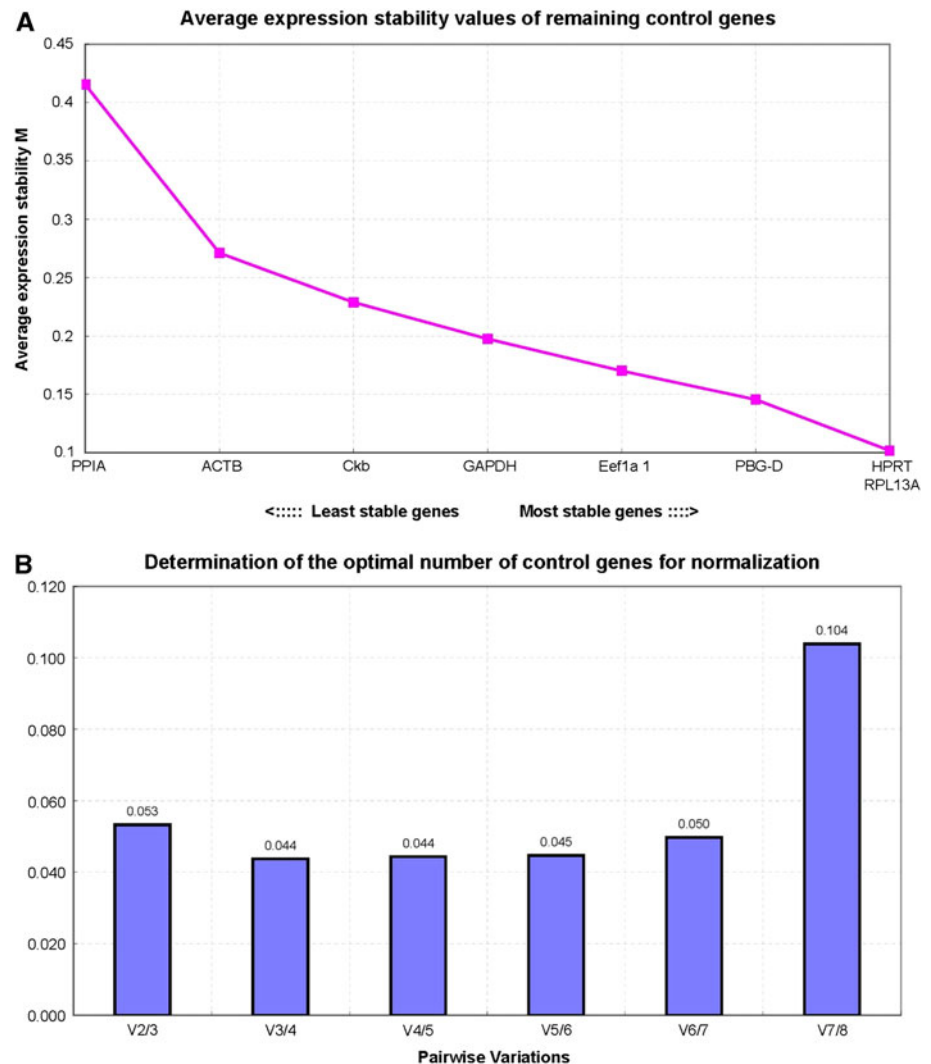
Discussion

RT-PCR is a predominant tool for gene expression studies. Selection of proper internal controls as housekeeping genes is a key point for ensuring credible expression of target genes. In our study, we have selected eight commonly used reference genes, namely, GAPDH, HPRT, PPIA, RPL13A, Actb, PBG-D, Eef1α1 and Ckb, which have both independent functions and constitutive expression in the cells. As far as we understand, this is the first study of the expression stability of candidate reference genes in vitro in ischemic and necrotic neural stem cell cultures. The final results indicate that a cautious choosing of the genes used for normalization is required for each experimental protocol.

Actb, GAPDH and PPIA are accepted as the classical internal controls in many researches [24], but recently have been shown to vary considerably between different cell types and tissues. We have found that these genes fluctuated greatly in neural stem cell hypoxic models and they may be co-expression under the hypoxic conditions which is very similar with other published results. It has been previously reported that PPIA protein is up-regulated in cortical neuronal cultures following several preconditioning treatments such as oxidative and ischemic injury [6]. Recently, it is also demonstrated that apoptosis-inducing

factor interacts with PPIA in the cytosol and the complex translocates to the nucleus, forms degradation, and initiates chromatinolysis. Gene deletion of PPIA decreases apoptosis-inducing factor translocation to the nucleus and reduces brain injury [30]. In recent study, frequently used housekeeping genes such as Actb and GAPDH were the most unstable genes investigated in hypoxia-cultured human chondrocytes [11]. The mRNA of Eef1α1 is the most abundantly expressed (1% of the total mRNA) in brain and is used as the proper housekeeping gene in several researches [13]. Factors such as warm ischemia and time at room temperature before extraction may influence the results of mRNA expression analysis. Eef1α1 has been adopted as a housekeeping gene when the effect of these factors on RNA integrity and mRNA expression levels is evaluated by RT-PCR [2]. And in the test of cerebral ischemia upregulates vascular endothelin ETB receptors in rat, Eef1α1 is also involved to serve as the reference gene [22]. Nevertheless, Eef1α1 was still not a trustworthy housekeeping gene due to our in vitro neural stem cell hypoxic models. The housekeeping genes Ckb expression is the highest in brain [27] and is also highly expressed among other genes in VM-derived neural stem cells [13]. In primary rat brain cell cultures, Ckb mRNA levels are found to be 15–17 times higher in astrocytes and oligodendrocytes than in embryonic neurons [16]. Numerous studies have demonstrated that hyperglycemia exacerbates neuronal damages initiated by cerebral ischemia. Increases in blood glucose and brain glucose concentration at the time of a cerebral ischemia event will worsen post-ischemic neurologic outcome [15]. PBG-D is a good control

Fig. 2 Gene expression stability and determination of the optimum number of genes for normalization of the candidate reference genes for the in vitro ischaemia model using geNorm analysis. **a** Expression stability plot showing average expression stability values *M*. **b** Pairwise variation analysis to determine the optimal number of reference genes for use in RT-PCR data normalization. The use of the two most stable genes is sufficient for accurate normalization (cut off 0.15)



gene based on the present case that hyperglycemia rapidly influences gene expression following cerebral ischemia [5]. Because of the high expression of *Ckb* in nerve system and related research on *PBG-D*, we included them as candidate housekeeping genes in this study, but the results suggest that the *Ckb* and *PBG-D* are not stable enough in the models for gene calibration. The results are also discussed with regard to the potential involvement of the expression of *Ckb* and *PBG-D* during hypoxia. It was reported that brain *HPRT* activity was unaffected by hypoxia–ischemia and could be taken as the conservation of brain purines after neurobiology injury [8], and this was contradict with our result between the different samples. *RPL13A* is a ribosomal protein that showed minimal variability in expression in different assay groups in the present study. Former research indicated that the most stable gene *RPL13A* covering a broad expression range, could be used as reference genes for relative gene quantification and normalization purposes in gene profiling studies of the rhesus monkey as determined by different reference gene

identification programs [1]. Other results also showed that *RPL13A* was the most stably expressed genes in the majority of the tissues examined [17]. Our results suggest that *RPL13A* is appropriate for being used as a reference gene in gene expression assay in rat neural stem cell hypoxic model [24].

The geNorm [29] and NormFinder [3] software are now widely used to make sure the stable reference genes from a set of candidate's genes with invariable expression [21, 25]. We have used this two analysis tools on genes identified as stable housekeeping genes in our in vitro rat neural stem cells hypoxic models and would like to accept the two validated genes *HPRT* and *RPL13A* together as the confident housekeeping gene in our future work.

In addition, to minimize the system errors for our RT-PCR reaction we firstly make sure the samples with equal quality were added to the final quantitation reaction, we carefully detected the extraction total RNA density and the cDNA density after RT reaction. This method, demonstrated by Schroder, can enhance the precision of

RT-PCR reaction [20]. To ensure the correct baseline or background for the density measurement, we adopted the reaction buffer for zero adjustment.

In conclusion, we selected eight commonly used reference genes including GAPDH, HPRT, PPIA, RPL13A, Actb, PBG-D, Eef1 α 1 and Ckb, and validate the 2 most stable housekeeping genes for neural stem cell in ischemic and necrotic cell cultures. This study provides the groundwork for a better understanding of the molecular changes occurring after hypoxic ischemic encephalopathy, and will help for future study of gene expression under hypoxic or ischemic condition in CNS.

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