Journal of Integrative Neuroscience

J. Integr. Neurosci. **2023**; 22(5): 125
https://doi.org/10.31083/j.jin2205125

Original Research

Hprt Serves as an Ideal Reference Gene for qRT-PCR Normalization in Rat DRG Neurons

Fenglan Liu[1,2,†], Yang Miao[3,†], Yaxian Wang[1], Qianqian Shan[1,2,*]

[1]Key Laboratory of Neuroregeneration of Jiangsu and Ministry of Education; Co-innovation Center of Neuroregeneration, Nantong University, 226001 Nantong, Jiangsu, China
[2]Department of Radiotherapy and Oncology, Affiliated Hospital of Nantong University, 226001 Nantong, Jiangsu, China
[3]Department of Pharmacy, The First People's Hospital of Yancheng, 224006 Yancheng, Jiangsu, China
*Correspondence: shanqianqian1986@126.com (Qianqian Shan)
[†]These authors contributed equally.
Academic Editor: Gernot Riedel
Submitted: 23 March 2023 Revised: 3 May 2023 Accepted: 24 May 2023 Published: 16 August 2023

Abstract

Objective: To identify suitable reference genes for gene expression studies in rat dorsal root ganglia (DRG) neurons. **Methods**: The raw cycle threshold (Ct) values of 12 selected reference genes were obtained via quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR) in neurons at different developmental stages or under different treatments. Two strategies were employed to screen the most stable reference genes: the genes were ranked according to the coefficient of biological variation and further validated using geNorm and NormFinder programs. The stable and unstable reference genes were subsequently used as internal controls to assess their effects on target gene expression. **Results**: All reference genes showed varying degrees of fluctuation in Ct values during the growth process of neurons or after different treatments. 18S ribosomal RNA (*Rn18s*) and β-actin (*Actb*) exhibited the most significant changes, while ubiquitin C (*Ubc*), hypoxanthine phosphoribosyl transferase (*Hprt*), and mitochondrial ribosomal protein L10 (*Mrpl10*) showed relatively minor changes. The most stable and unstable genes obtained by different evaluation methods varied slightly. Overall, *Actb* was found to be the most unstable reference gene, while *Hprt* was the relatively most stable reference gene. The use of unstable reference genes *Actb* and ankyrin repeat domain 27 (*Ankrd27*) as internal controls led to high variability within the control group, ultimately affecting the determination of target gene expression. In contrast, the stable reference gene *Hprt* had small inter-assay variation and high stability. **Conclusions**: Our observations indicate that *Hprt* is a proper endogenous reference gene for qRT-PCR analysis in rat DRG neurons and thus provides a critical molecular basis for the genetic characterization in neurological disorders.

Keywords: DRG neuron; qRT-PCR; reference gene; expression stability; *Hprt*

1. Introduction

The mammalian nervous system perceives sensory information and sensations, integrates and analyzes received signals, and generates rapid and appropriate responses. The stimulus-response coordination of the nervous system largely depends on the proper functioning of neurons, specialized for signal conduction and network integration. Altered gene expression patterns in neurons may cause neuronal dysfunction and are involved in various neurodevelopmental and neurological disorders [1–3]. Genetic manipulation of neurons offers promising therapeutic strategies [4]. The examination of neuronal gene expression profiling helps to elucidate dysregulated genes and decipher effective molecular targets.

Recent advances in high-throughput genome analysis methods, such as microarray and sequencing, facilitate the comprehensive description of genome-wide expression under physiological and pathological conditions. The single-cell sequencing era further permits the determination of gene abundances in single cells instead of whole tissues and organs [5]. Genetic changes in neurons have thus been explored in many neurological diseases, e.g., Alzheimer's disease, Parkinson's disease, amyotrophic lateral sclerosis, pain, and traumatic nerve injury [6–10]. These technologies are productive and cost-effective for large-scale gene profiling. To add validity to high-throughput data, quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR) has been used as a routine method to determine the abundance of screened genes [11–13]. Besides providing supplementary data for high-throughput analysis, precise quantification of certain genes using qRT-PCR is substantial to determine the effects of gene editing prior to functional examinations.

The qRT-PCR method captures and monitors the emission of fluorescent probes binding to double-stranded DNA and measures cycle threshold (Ct) values, which are the numbers of polymerase chain reaction (PCR) cycles required for the fluorescence signals to cross the threshold base [14]. Obtained Ct values are affected by many factors, such as template quantity and quality, reagent specificity, enzyme activity, master mixes, reaction efficiency, and technological variability. Amplifying an extra reference gene whose expression is generally constant across

diverse biological conditions together with target genes allows gene expression normalization and minimizes bias of raw Ct values. Reference genes are genes that are involved in the maintenance of the fundamental functions of cells. They are considered ubiquitously and steadily expressed in all tissue and organs and thus are commonly used as internal controls in qRT-PCR [15,16]. However, emerging studies demonstrate that many reference genes show varied expression levels under different biological and experimental conditions [17,18]. For instance, reference genes that are found to be invariant in blood or human CD4 T cell cultures are unstably expressed in human peripheral blood mononuclear cell cultures [19].

Given that reference genes have surprisingly diverse abundances in different situations, it is necessary to adopt experimental condition-specific reference genes to increase the accuracy of transcript normalization [20]. Herein, taking the importance of selecting stably expressed reference genes in neurons in consideration, we collected rat dorsal root ganglia (DRG) tissues, isolated and cultured primary DRG neurons, and evaluated the expression stabilities of nine commonly used reference genes, including *Actb* (β-actin), *CypA* (cyclophilin A), *Gapdh* (glyceraldehydes-3-phosphate dehydrogenase), *Hprt* (hypoxanthine phosphoribosyl transferase), *Pgk1* (phosphoglycerate kinase 1), *Rn18S* (18S ribosomal RNA), *Tbp* (TATA box binding protein), Ubc (ubiquitin C), and *Ywhaz* (tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta); as well as three reference genes previously identified by Gambarotta *et al.* [21], including *Ankrd27* (ankyrin repeat domain 27), *Mrpl10* (mitochondrial ribosomal protein L10), and *Rictor* (rapamycin-insensitive companion of mTOR, complex 2). Raw Ct values of these reference genes in neurons under distinctive conditions as well as bioinformatic data from commercially available software algorithms geNorm and NormFinder were jointly analyzed to rank the expression stabilities of these genes and to select ideal reference genes for qRT-PCR normalization in neurons.

2. Materials and Methods

2.1 Neuron Isolation, Culture, and Transfection

A total of 18 adult Sprague-Dawley rats were utilized in this experiment. After the rats were euthanized by CO_2 asphyxiation, DRG tissues were obtained and carefully dissected in Hank's balanced salt solution (HBSS). Subsequently, the tissues were enzymatically digested using collagenase type I and trypsin, then triturated in a Neurobasal medium supplemented with 2% B27, 1% glutamine, and 1% penicillin-streptomycin (Thermo Fisher Scientific, Waltham, MA, USA). Cells were passed through a 70 µm strainer, resuspended, and seeded on ploy-L-Lysine-coated 24-well plates. To ensure the absence of contamination, mycoplasma testing was performed on the cells used. Purification of neurons was attained by a 48-hour treatment using cytarabine (1:1000, 147-94-4, Sigma, St Louis, MO, USA) to eliminate non-neuronal cells as previously described [22]. The purity of the neurons was confirmed by staining with a neuron marker, βIII tubulin (Ab18207, Abcam, Cambridge, UK). Next, the purified neurons were resuspended and replanted into 24-well plates with or without coverslips. Previous studies have shown that primary cultured hippocampal neurons exhibit several typical developmental stages during growth [23]. Combining our previous preliminary experiment results in DRG neuron culture; cells were collected at 12, 24, 48, and 72 h after replantation for immunostaining or RNA extraction. All experiments were conducted with three biological replicates under the same conditions.

To knock down long non-coding (lnc) 364 in neurons, short interfering RNA (siRNA) segments targeting lnc364 (siRNA-364-1, GCATCCACAATGAATTGAT; siRNA-364-2, GGTCCAAACAGGAGAACAT; and siRNA-364-3, CCATATAGTTTGGCTACTG) or a negative siRNA control consisting of a random sequence (Ribobio, Guangzhou, Guangdong, China) were transfected using Lipofectamine RNAiMAX transfection reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions. Briefly, the purified neurons were replanted and incubated with the transfection reagent for 16 h, after which the medium was renewed, and the cells were left to culture for an additional 24 h prior to being harvested for subsequent analysis.

2.2 Immunostaining and Morphometric Analysis

Cultured neurons were fixed with 4% paraformaldehyde (PFA, Xilong Scientific, Guangzhou, Guangdong, China), blocked with Immunol Staining Blocking Buffer (Beyotime, Shanghai, China), and stained with βIII tubulin (Ab18207, Abcam, Cambridge, UK). Neuronal soma and neurites were visualized under a Zeiss Axio Imager M2 microscope (Carl Zeiss Microscopy GmbH, Jena, Germany). The longest and total lengths of neurites were quantified with Image J (Image J 1.50i, NIH, Bethesda, MD, USA). To measure the detailed topologies of the neurites at different developmental stages, we performed sholl analysis on βIII tubulin-labeled neurons with the aid of Image J software. Briefly, a set of concentric circles with increasing radii (20 µm apart) were overlaid on the neuron body, and the number of neurite branches intersecting each circle was counted to obtain the distribution pattern of neurites in different regions [24].

2.3 RNA Extraction and qRT-PCR Examination

RNA was extracted from cultured neurons using TRIzol regent (Invitrogen, Carlsbad, CA, USA) and quantified using a Nanodrop 1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). Complementary DNA (cDNA) synthesis for the validation experiments utilized 1 ug of RNA. After converting RNA to cDNA, qRT-

Table 1. Primer sequences of candidate reference genes.

Symbol	Accession number		Primer sequences (5′-3′)	Amplicon size	Efficiency	Reference
Actb	NM_031144.2	Sense Antisense	AGGCCAACCGTGAAAAGATG ACCAGAGGCATACAGGGACAA	101	97%	Martínez-Beamonte *et al*., 2011 [26]
Ankrd27	NM_001271264.1	Sense Antisense	CCAGGAGACAGAACACGAGG CCCCTGGGTTAATGAGGCAA	119	101%	*
CypA	NM_017101.1	Sense Antisense	TATCTGCACTGCCAAGACTGAGTG CTTCTTGCTGGTCTTGCCATTCC	126	100%	*
Gapdh	NM_017008.4	Sense Antisense	CCACCAACTGCTTAGCCCCC GCAGTGATGGCATGGACTGTGG	91	101%	Gambarotta *et al*., 2014 [21]
Hprt	NM_012583.2	Sense Antisense	CTCATGGACTGATTATGGACAGGAC GCAGGTCAGCAAAGAACTTATAGCC	123	99%	Peinnequin *et al*., 2004 [27]
Mrpl10	NM_001109620.1	Sense Antisense	CTCCTCCCAAGCCCCCCAAG CAGACAGCTATCATTCGGTTGTCCC	97	90%	Gambarotta *et al*., 2014 [21]
Pgk1	NM_053291.3	Sense Antisense	GCAGATTGTTTGGAACGGTCC TAGTGATGCAGCCCCTAGACGT	113	100%	Martínez-Beamonte *et al*., 2011 [26]
Rictor	XM_001055633.3	Sense Antisense	TCCGAATACGAGGGCGGAA AGATGGCCCAGCTTTCTCATA	142	95%	*
Rn18s	X01117.1	Sense Antisense	ACTCAACACGGGAAACCTCA AATCGCTCCACCAACTAAGA	114	102%	Bangaru *et al*., 2012 [29]
Tbp	NM_001004198.1	Sense Antisense	TGGGATTGTACCACAGCTCCA CTCATGATGACTGCAGCAAACC	131	102%	Seol *et al*., 2011 [28]
Ubc	NM_017314.1	Sense Antisense	ATCTAGAAAGAGCCCTTCTTGTGC ACACCTCCCCATCAAACCC	51	102%	Bangaru *et al*., 2012 [29]
Ywhaz	NM_013011.3	Sense Antisense	GATGAAGCCATTGCTGAACTTG GTCTCCTTGGGTATCCGATGTC	117	94%	Seol *et al*., 2011 [28]

* Designed by Primer Express® software version 3.0 (Applied Biosystems, Foster City, CA, USA).

PCR was performed using SYBR Premix Ex Taq (TaKaRa, Dalian, Liaoning, China) on a StepOne qRT-PCR System (Applied Biosystems, Foster City, CA, USA). Additionally, referring to previous studies [25], we prepared cDNA from a mixed RNA sample, designated as pool cDNA, for subsequent analysis of primer amplification efficiency. Raw Ct values of reference genes were recorded. The relative abundance of lnc364 was quantified using the comparative $2^{-\Delta\Delta Ct}$ method. The primers used are listed in Table 1 (Ref. [21,26–29]).

2.4 Bioinformatic Analysis

Three replicates of each sample were analyzed to obtain an average Ct value, and the coefficient of variation (CV) was calculated for each group. The percentage CV was obtained by dividing the standard deviation by the mean Ct, which was used to compare variation among the 12 reference genes. To confirm the results, geNorm (v3.4; https://genorm.cmgg.be/) and NormFinder (v19; https://www.moma.dk/software/normfinder) analyses were conducted using the Microsoft Excel 2007 Visual Basic plug-in (Microsoft Corporation, Redmond, WA, USA). The geNorm analysis calculated the gene expression stability value (M), which was negatively correlated with gene expression stability, and the normalization factor (pairwise variation value, Vn/n + 1) was calculated based on the geometric mean of multiple reference genes. A Vn/n + 1 value less than 0.15 indicated that no additional reference genes were required [29]. NormFinder analysis was applied to evaluate intra-group and intergroup variations and provide expression stability values [29].

2.5 Statistical Analysis

Statistical analysis was performed with GraphPad Prism 6.0 (GraphPad software, La Jolla, CA, USA) using one-way analysis of variance followed by a Dunnett post hoc test. A *p*-value less than 0.05 was considered a significant difference.

3. Results

3.1 Morphological Observations of Rat DRG Neurons

Primary neurons isolated from rat DRG tissues were seeded onto a culture plate pre-coated with poly-L-Lysine and observed at 12, 24, 48, and 72 h after cell culture. Immunostaining with neuronal marker βIII tubulin showed that at 12 h after cell seeding, neurons attached to the

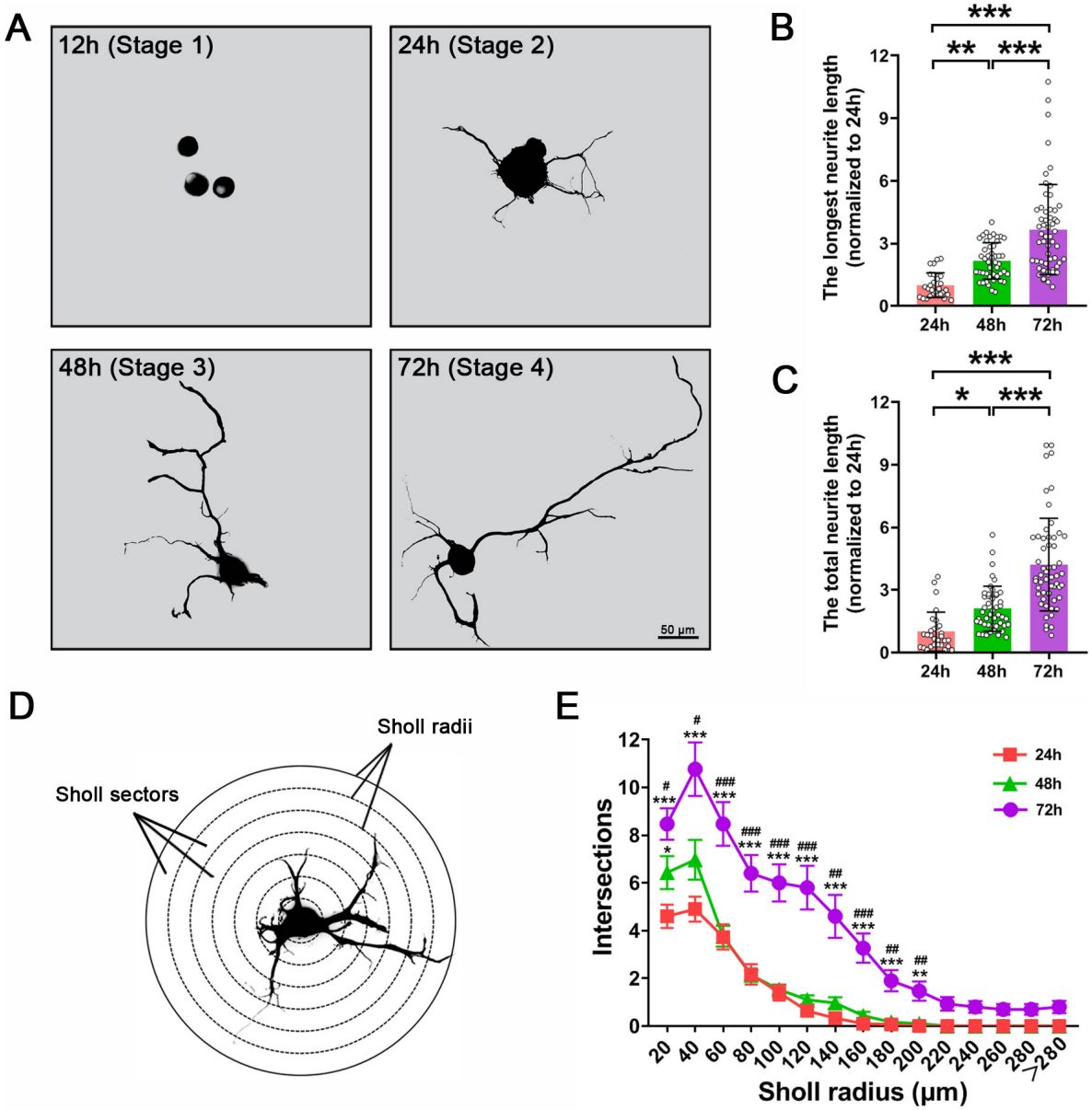

Fig. 1. Different developmental stages of rat dorsal root ganglia (DRG) neurons during a 72-hour culture. (A) Immunostaining of DRG neurons cultured for 12, 24, 48, and 72 h. Scale bar represents 50 μm. (B) Summarized longest neurite length. **, *p*-value < 0.01; ***, *p*-value < 0.001. (C) Summarized total neurite length. *, *p*-value < 0.05; ***, *p*-value < 0.001. (D) Diagram of neurite branches intersecting each circle. (E) Quantification of intersections by Sholl analysis. *vs*. 24 h, *, *p*-value < 0.05; **, *p*-value < 0.01; ***, *p*-value < 0.001; *vs*. 48 h, #, *p*-value < 0.05; ##, *p*-value < 0.01; ###, *p*-value < 0.001. All data were generated from three paired experiments (n = 3).

substrate. Some neurites appeared after a 24-hour culture period, and prolonged neurites were observed 48 h after culture. Notably, among extended neurites, one neurite seemed to be much longer than others. The length of the longest neurite was observed to be further increased after culturing for additional 24 h and could be clearly distinguished as the neuronal axon (Fig. 1A). DRG neurons exhibited similar neurite extension patterns as cultured hippocampal neurons [23]. Therefore, according to the separation of developmental stages of hippocampal neurons, DRG neurons cultured for 12, 24, 48, and 72 h are designated as stages 1, 2, 3, and 4, respectively. Comparison of the neurites of neurons in different states demonstrated that the length of the longest neurite increased to nearly 2 folds from stage 2 to stage 3 and to more than 3 folds when neurons entered stage 4 (Fig. 1B). The lengths of total neurites were also elevated from stage 2 to stage 3 and stage 4 (Fig. 1C). In addition, sholl analysis showed that the neurite branches of neurons in stage 4 were significantly increased compared with other stages (Fig. 1D,E)

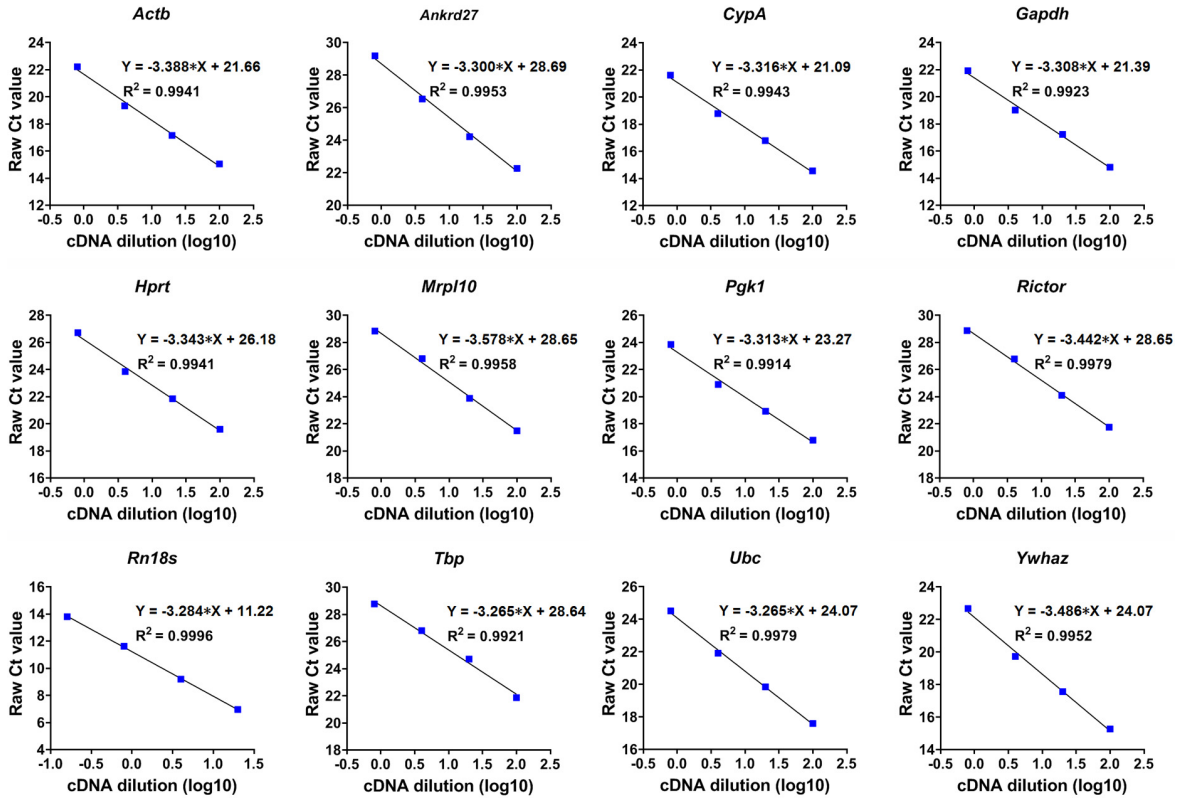

Fig. 2. Raw cycle threshold (Ct) values of reference genes in quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR) serial dilution. The Ct values of reference genes *Actb*, *Ankrd27*, *CypA*, *Gapdh*, *Hprt*, *Mrpl10*, *Pgk1*, *Rictor*, *Rn18S*, *Tbp*, *Ubc*, and *Ywhaz* in serial dilution of the pool cDNA sample. The slope and linear correlation coefficient R^2 of each reference gene are displayed. All data were generated from three paired experiments (n = 3).

3.2 Expression Levels of Candidate Reference Genes in Neurons

Raw Ct values of a total of 12 reference genes, including *Actb* (*β*-actin), *Ankrd27* (ankyrin repeat domain 27), *CypA* (cyclophilin A), *Gapdh* (glyceraldehydes-3-phosphate dehydrogenase), *Hprt* (hypoxanthine phosphoribosyl transferase), *Mrpl10* (mitochondrial ribosomal protein L10), *Pgk1* (phosphoglycerate kinase 1), *Rictor* (rapamycin-insensitive companion of mTOR, complex 2), *Rn18S* (18S ribosomal RNA), *Tbp* (TATA box binding protein), *Ubc* (ubiquitin C), and *Ywhaz* (tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta), were examined after the success culture of primary DRG neurons. First, the amplification efficiency of each primer pair was analyzed by producing a 10-fold serial dilution of the pool cDNA sample. The amplifications of reference genes showed that all tested reference genes had linear correlation coefficients (R^2) that were higher than 0.99 (Fig. 2). The efficiencies calculated based on slopes using the equation $E = -1 + 10^{(-1/slope)}$ showed the amplification efficiencies ranged from 90% to 110% (Table 1). It indicated that these primers had high efficacy and quality, meeting the standards for qRT-PCR. These primers were thus used to determine the raw Ct values of reference genes in cultured neurons.

The Ct values are inversely proportional to gene abundances. *Rn18s* had the lowest Ct values (Ct values = 7.10~9.84) and the highest expression levels in neurons, while *Ankrd27*, *Mrpl10*, *Rictor*, and *Tbp* had the highest Ct values and relatively lower abundances (Fig. 3). Additionally, the Ct values of the same reference gene in neurons cultured under different conditions were distinctive. For instance, *Actb* had a raw Ct value of 17.63 in DRG neurons cultured for 12 h but an increased Ct value of 18.03 in neurons cultured for 24 h and slightly decreased Ct values of 16.89 and 16.99 in neurons cultured for 48 and 72 h, respectively. The Ct value of *Actb* in neurons exhibited a much larger change after cell transfection. Neurons transfected with control siRNA had a Ct value of 16.81 for *Actb*, while neurons transfected with siRNA against lnc364 had a Ct value of 19.61. Similar to *Actb*, the raw Ct values of other reference genes in neurons under different conditions were not immutable. Among these reference genes, the raw Ct values of *Hprt* had the smallest ΔCt and the lowest extent of dispersion (Ct values = 21.96~23.79), demonstrating the high stability of *Hprt*.

3.3 Expression Stabilities of Candidate Reference Genes

The expression stability of the 12 reference genes was first evaluated by ranking them based on their global biolog-

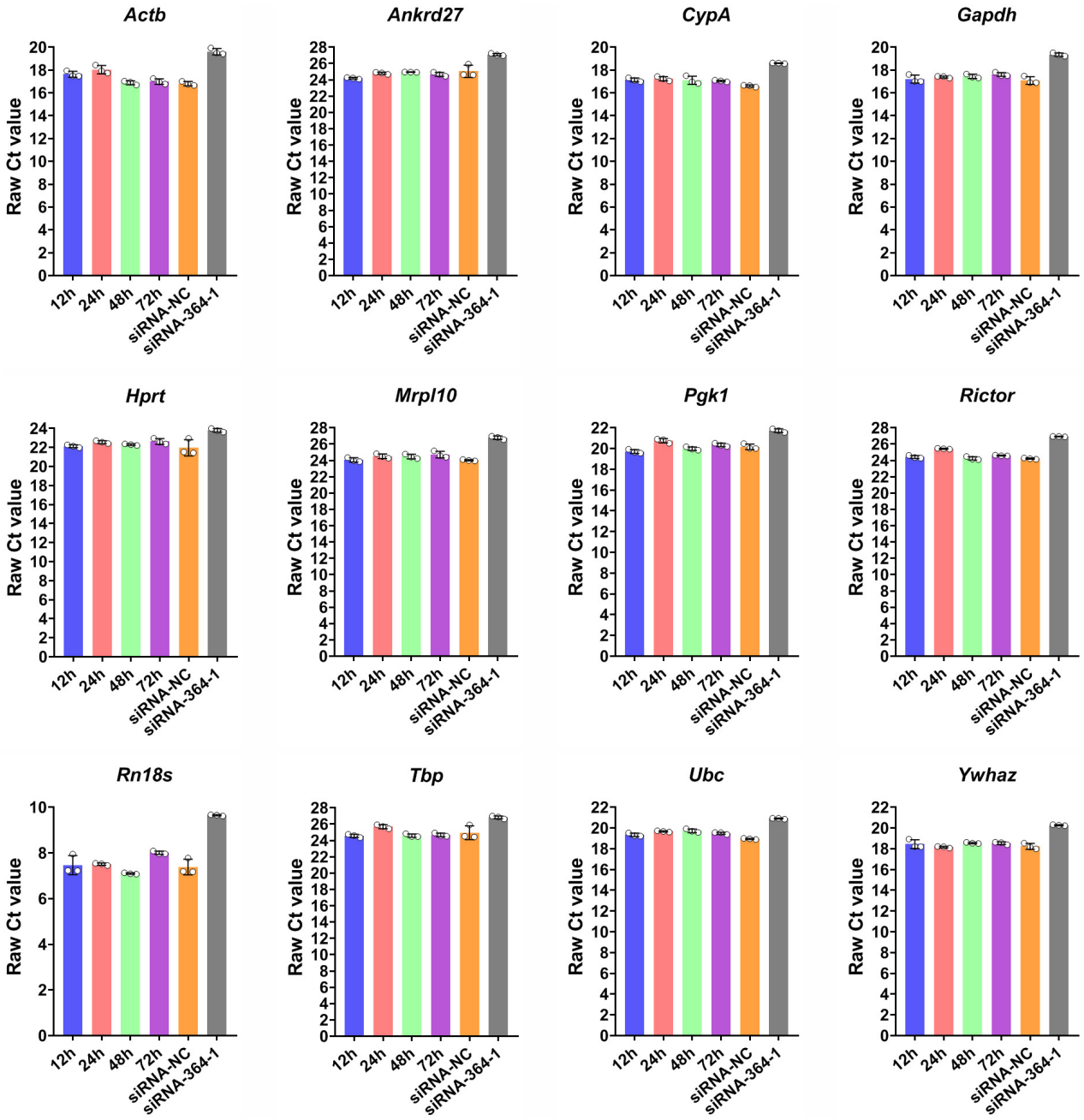

Fig. 3. Raw Ct values of reference genes in neurons under distinctive conditions. The Ct values of reference genes *Actb*, *Ankrd27*, *CypA*, *Gapdh*, *Hprt*, *Mrpl10*, *Pgk1*, *Rictor*, *Rn18S*, *Tbp*, *Ubc*, and *Ywhaz* in neurons cultured for 12, 24, 48, and 72 h and neurons transfected with siRNA against lnc364 (siRNA-364-1) or a siRNA control (siRNA-NC). All data were generated from three paired experiments (n = 3).

ical coefficient of variation (CV) in neurons under various developmental stages or treatments. Table 2 illustrates the ranked results, which indicate that *Hprt* had the most stable expression among the tested genes, whereas *Rn18s* showed the highest inter-experiment variation.

Bioinformatic tools geNorm and NormFinder were further applied to determine the expression stabilities of these reference genes. The geNorm algorithm calculates the average pair-wise variation based on the principle that the ratio of the expression levels of two ideal reference genes shall be consistent. Genes with expression variation M values greater than 1.5 are considered as erratically expressed and hence are not appropriate for being used as reference genes. In cultured neurons, variation M values ranged from 0.10 to 0.45, with *Actb* having the highest M value and being the least stable gene and *CypA*/*Ubc* having the lowest M value and being the most stable gene (Fig. 4A). Pairwise variation Vn/n + 1 was also calculated to determine the optimal numbers of reference genes for gene quantification, in which n refers to the number of reference genes. The cal-

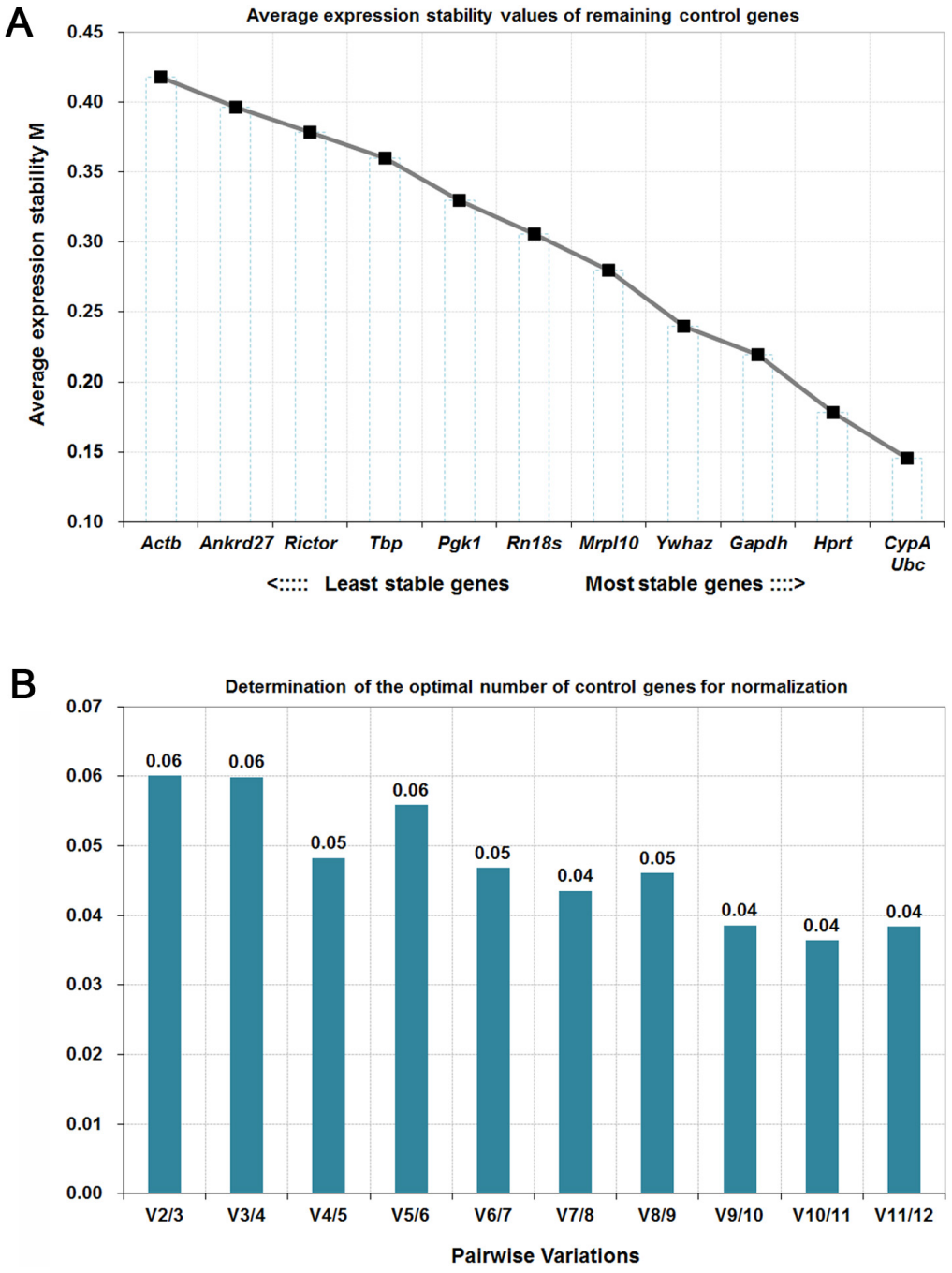

Fig. 4. Expression stabilities of reference genes in neurons determined by geNorm analysis. (A) Average expression stability of reference genes *Actb*, *Ankrd27*, *CypA*, *Gapdh*, *Hprt*, *Mrpl10*, *Pgk1*, *Rictor*, *Rn18S*, *Tbp*, *Ubc*, and *Ywhaz*. (B) Pairwise variations of reference genes.

culated pairwise variation value V2/3 was 0.06, far below the default limit of Vn/n + 1 = 0.15, suggesting that it is unnecessary to increase the number of reference genes for elevating expression stability (Fig. 4B).

The NormFinder program directly measures the stability value of each gene. In consistent with the stability ranking generated with geNorm, *Actb* and *Ankrd27* were the two most unstable reference genes. *Hprt*, a reference gene that ranked third place using geNorm analysis, had the lowest value using NormFinder (Fig. 5). Together with the lowest CV values of raw Ct of *Hprt* in neurons cultured for different time periods and subjected to siRNA transfection, it is considered that *Hprt* is the most stable reference gene in neurons.

3.4 Influences of a Suitable Selection of Reference Genes

Stably expressed reference gene *Hprt*, as well as unstably expressed reference genes *Actb* and *Ankrd27*, were

Table 2. Gene expression of 12 reference genes ranked according to the biological coefficient of variation.

Gene symbol	Mean Ct	SD	CV(%)	Rank
Hprt	22.56	0.60	2.65	1
Ubc	19.69	0.60	3.07	2
Pgk1	20.45	0.65	3.20	3
Tbp	25.22	0.82	3.24	4
CypA	17.29	0.62	3.59	5
Ankrd27	25.13	0.92	3.65	6
Mrpl10	24.77	0.94	3.78	7
Ywhaz	18.70	0.72	3.83	8
Rictor	24.98	0.96	3.85	9
Gapdh	17.68	0.76	4.33	10
Actb	17.66	0.97	5.52	11
Rn18s	7.85	0.85	10.81	12

Ct, cycle threshold; SD, standard deviation; CV, coefficient of variation.

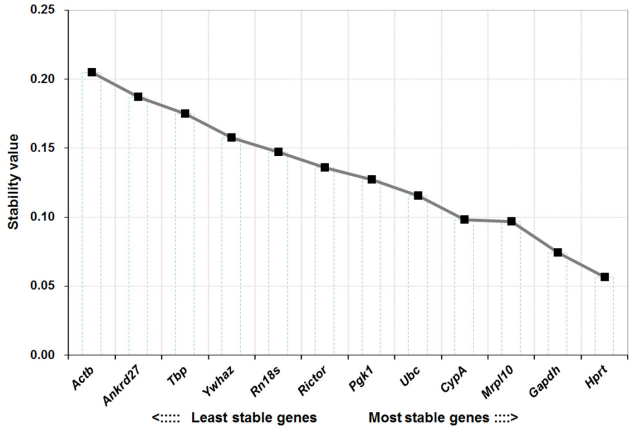

Fig. 5. Expression stabilities of reference genes in neurons determined by NormFinder analysis. Stability values of reference genes *Actb*, *Ankrd27*, *CypA*, *Gapdh*, *Hprt*, *Mrpl10*, *Pgk1*, *Rictor*, *Rn18S*, *Tbp*, *Ubc*, and *Ywhaz*.

utilized as internal controls, respectively, to perform qRT-PCR and to determine the expression levels of target gene lnc364. Besides siRNA-364-1, siRNA-364-2 and siRNA-364-3, two other siRNA segments against lnc364 were transfected into cultured neurons. The relative expression levels of lnc364 normalized with *Actb* exhibited a significant decrease after being transfected with siRNA-364-1. By contrast, the *Actb*-normalized expression levels of lnc364 in neurons transfected with siRNA-364-2 and siRNA-364-3 were slightly decreased but not significantly different from the abundance of lnc364 in control neurons. Using *Ankrd27*, another unstable reference gene in neurons as the reference gene, the expression variation of lnc364 in neurons transfected with control siRNA was robustly higher. Hence, there existed no significant differences in lnc364 abundances in neurons transfected with siRNA segments against lnc364 and control neurons. Smaller variation was observed when using stable expressed *Hprt* as the reference gene. The relative expression level of lnc364 normalized with *Hprt* showed a nearly 70.0% decrease after siRNA-364-1 transfection with a p-value less than 0.001. Moreover, neurons transfected with siRNA-364-2 also had a significantly diminished lnc364 expression with a *p*-value less than 0.05 (Fig. 6). These findings demonstrate that siRNA-induced expression reduction is most apparent when normalizing to the most stable reference gene *Hprt*.

4. Discussion

qRT-PCR is a highly rapid, reproducible, and sensitive method for the detection and quantification of genes of interest. In qRT-PCR, the selection of suitable reference genes increases the precise and reliable quantification of gene abundances. Previously, we have collected rat DRG tissues, distal sciatic nerve segments, and gastrocnemius muscles after sciatic nerve injury and found that diverse tissues have their own stably expressed reference genes. *Tbp*, *Mrp10*, and *Hprt* are separately identified as suitable reference genes in neuronal soma-containing tissues, nerve stumps, and target muscles during the peripheral nerve repair and regeneration process [30,31]. Reference genes *Ankrd27*, *Ubc*, and *Tbp* are found to be stably expressed in the spinal cord after injury to central nerves [32]. Additionally, in the central nervous system, different reference genes are screened in the denervated granule cell layer and outer molecular layer of the dentate gyrus after mouse brain injury [33]. Besides the identification of diverse stably expressed reference genes in different tissues and organs, a recent study demonstrates that unique reference genes should be used as internal controls in skeletal muscle in diverse body sections and murine C2C12 myoblast cells, a cell line commonly used for the *in vitro* investigation of skeletal muscle [34]. Nerve tissues contain a large number of different cell types. An earlier study in our group, using single-cell sequencing, shows that rat DRG tissues contain neurons, satellite glial cells, Schwann cells, fibroblasts, erythrocytes, vascular smooth muscle cells, pericytes, endothelial cells, and immune cells [35]. The diversity of cell populations tells us that for the investigation of the nervous system, it may be necessary to determine proper reference genes for specific cell types, especially neurons.

DRG tissues, also called spinal ganglia, are nodule-like structures localized on the posterior roots of the spinal cord [36]. The anatomical localization of DRG tissues makes it convenient to collect DRG tissues. DRG neurons are thus commonly gathered and cultured for physiological and pathological investigations, such as proprioception, chronic pain, and diabetic neuropathy [37,38]. Furthermore, anatomically, axons of DRG neurons are bifurcated into central and peripheral branches. The unique features of DRG neurons make them suitable models for determining molecular changes and the intrinsic growth capacity of neurons after central and peripheral branch injury [39]. In

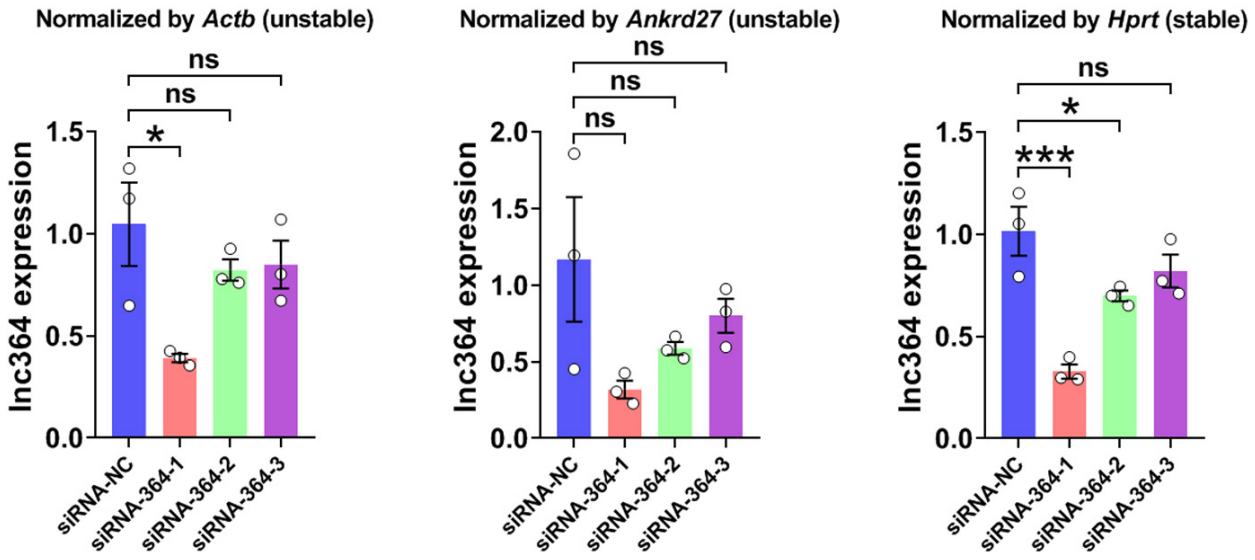

Fig. 6. Quantification of lnc364 abundance in neurons transfected with siRNA against lnc364. The expression levels of lnc364 in neurons transfected with siRNA segments against lnc364 (siRNA-364-1, siRNA-364-2, and siRNA-364-3) or a siRNA control (siRNA-NC) using *Actb*, *Ankrd27*, and *Hprt* as reference genes. *, p-value < 0.05; ***, p-value < 0.001; ns, not significant. All data were generated from three paired experiments (n = 3).

view of the wide application of DRG neurons, herein, we measured the expression changes and expression stabilities of many frequently used reference genes in DRG neurons.

Algorithms geNorm, and NormFinder calculates gene stabilities based on different calculation models. The former obtains the mostly stably expressed genes by excluding unstably expressed genes stepwise, while the latter straightforwardly calculated the most stably expressed genes based on intra- and inter-group variations [40,41]. Similar to observations from other tissues and cell cultures, in cultured neurons, geNorm and NormFinder-determined gene rankings are not completely the same [42,43]. *Hprt* displays minimal changes in raw Ct values (Ct values = 21.96~23.79) and has the smallest biological coefficient of variation (CV) among all tested genes. It was identified as one of the most stable genes by both geNorm and NormFinder analyses, highlighting its suitability as a reference gene for quantifying neuronal gene expression. References genes for many cell populations, such as macrophages, osteoblasts, osteoclasts, gastric cells, and hepatoma cells, have been discovered [44–46]. Our current study establishes a reference gene in cultured DRG neurons and thus provides an important basis for neurological studies.

The application of reference genes is particularly essential for the precise quantification of genes with low abundances. lncRNAs, although they function as important regulators in the nervous system, often have low abundances in the genome [47,48]. After the identification of unstable and stable reference genes, we evaluated the relative abundances of lnc364 in cultured DRG neurons. Lnc364 was dysregulated in neurons after peripheral and central branch injury, and neurons were transfected with siRNA segments against lnc364 to determine the effect of silencing lnc364 on neurite outgrowth and axon regeneration (unpublished data). With *Hprt* as the reference gene, siRNA-364-2 induces a significant reduction of lnc364 and can be used as another independent siRNA segment to eliminate the possibility of off-target effects. These results fully demonstrate the effects of reference genes normalization on gene expression levels and indicate the importance of using stable reference genes as internal controls. Notably, the direct measurement of raw Ct values shows that gene abundances of reference genes are altered after transfection, telling that genetic manipulation may elicit robust cellular changes and influence reference gene expression. These novel findings further indicate that the expression levels of reference genes are not stable but highly related to cellular states.

In the present study, we calculate the expression variations of a large number of commonly used reference genes and identify *Hprt* as an ideal reference gene in cultured DRG neurons. However, it should be noted that the tools used in this study are not suitable for the de novo identification of genes with calibration potential, and therefore it is possible that this approach may have overlooked other viable reference genes in neurons. By employing alternative methods like single-cell RNA sequencing or genomic screening, it may be possible to identify other genes as internal controls, and this could contribute to further investigations of the nervous system.

5. Conclusions

In summary, in this study, we conducted a systematic analysis of the stability of 12 reference genes in neurons across various developmental stages or after different treatments. Ultimately, we discovered that *Hprt* is a suitable endogenous reference gene for qRT-PCR analysis in rat DRG neurons. This finding offers crucial molecular groundwork for the genetic characterization of neurological disorders.

Availability of Data and Materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Author Contributions

Conceived and designed the experiments: QS; Performed the experiments: FL, YM; Analyzed the data: FL, YM, YW and QS; Contributed to applying reagents/materials/analysis tools: YW, QS; Wrote the manuscript: FL, QS. All authors contributed to editorial changes in the manuscript. All authors read and approved the final manuscript. All authors have participated sufficiently in the work and agreed to be accountable for all aspects of the work.

Ethics Approval and Consent to Participate

This study was approved by the Administration Committee of Experimental Animals, Jiangsu Province, China (approval No. IACUC20220311-002).

Acknowledgment

The authors would like to thank Sheng Yi of Nantong University (China) for her assistance in refining the language in this manuscript.

Funding

This study was supported by Natural Science Research Project of Nantong Health and Family Planning Commission [QA2019058].

Conflict of Interest

The authors declare no conflict of interest.

References

[1] Wang Y, Chakravarty P, Ranes M, Kelly G, Brooks PJ, Neilan E, *et al*. Dysregulation of gene expression as a cause of Cockayne syndrome neurological disease. Proceedings of the National Academy of Sciences of the United States of America. 2014; 111: 14454–14459.

[2] Klingler E, Francis F, Jabaudon D, Cappello S. Mapping the molecular and cellular complexity of cortical malformations. Science. 2021; 371: eaba4517.

[3] Zalocusky KA, Najm R, Taubes AL, Hao Y, Yoon SY, Koutsodendris N, *et al*. Neuronal ApoE upregulates MHC-I expression to drive selective neurodegeneration in Alzheimer's disease. Nature Neuroscience. 2021; 24: 786–798.

[4] Kampmann M. CRISPR-based functional genomics for neurological disease. Nature Reviews Neurology. 2020; 16: 465–480.

[5] Tang F, Barbacioru C, Wang Y, Nordman E, Lee C, Xu N, *et al*. mRNA-Seq whole-transcriptome analysis of a single cell. Nature Methods. 2009; 6: 377–382.

[6] Mathys H, Davila-Velderrain J, Peng Z, Gao F, Mohammadi S, Young JZ, *et al*. Single-cell transcriptomic analysis of Alzheimer's disease. Nature. 2019; 570: 332–337.

[7] Agarwal D, Sandor C, Volpato V, Caffrey TM, Monzón-Sandoval J, Bowden R, *et al*. A single-cell atlas of the human substantia nigra reveals cell-specific pathways associated with neurological disorders. Nature Communications. 2020; 11: 4183.

[8] Liu W, Venugopal S, Majid S, Ahn IS, Diamante G, Hong J, *et al*. Single-cell RNA-seq analysis of the brainstem of mutant SOD1 mice reveals perturbed cell types and pathways of amyotrophic lateral sclerosis. Neurobiology of Disease. 2020; 141: 104877.

[9] Wang K, Wang S, Chen Y, Wu D, Hu X, Lu Y, *et al*. Single-cell transcriptomic analysis of somatosensory neurons uncovers temporal development of neuropathic pain. Cell Research. 2021; 31: 904–918.

[10] Renthal W, Tochitsky I, Yang L, Cheng YC, Li E, Kawaguchi R, *et al*. Transcriptional Reprogramming of Distinct Peripheral Sensory Neuron Subtypes after Axonal Injury. Neuron. 2020; 108: 128–144.e9.

[11] VanGuilder HD, Vrana KE, Freeman WM. Twenty-five years of quantitative PCR for gene expression analysis. BioTechniques. 2008; 44: 619–626.

[12] Benes V, Castoldi M. Expression profiling of microRNA using real-time quantitative PCR, how to use it and what is available. Methods. 2010; 50: 244–249.

[13] Zucha D, Kubista M, Valihrach L. Tutorial: Guidelines for Single-Cell RT-qPCR. Cells. 2021; 10: 2607.

[14] Nolan T, Hands RE, Bustin SA. Quantification of mRNA using real-time RT-PCR. Nature Protocols. 2006; 1: 1559–1582.

[15] Zhu J, He F, Song S, Wang J, Yu J. How many human genes can be defined as housekeeping with current expression data? BMC Genomics. 2008; 9: 172.

[16] Eisenberg E, Levanon EY. Human housekeeping genes, revisited. Trends in Genetics. 2013; 29: 569–574.

[17] Schmittgen TD, Zakrajsek BA. Effect of experimental treatment on housekeeping gene expression: validation by real-time, quantitative RT-PCR. Journal of Biochemical and Biophysical Methods. 2000; 46: 69–81.

[18] de Jonge HJM, Fehrmann RSN, de Bont ESJM, Hofstra RMW, Gerbens F, Kamps WA, *et al*. Evidence based selection of housekeeping genes. PLoS ONE. 2007; 2: e898.

[19] Dheda K, Huggett JF, Bustin SA, Johnson MA, Rook G, Zumla A. Validation of housekeeping genes for normalizing RNA expression in real-time PCR. BioTechniques. 2004; 37: 112–112–4, 116, 118–119.

[20] Guénin S, Mauriat M, Pelloux J, Van Wuytswinkel O, Bellini C, Gutierrez L. Normalization of qRT-PCR data: the necessity of adopting a systematic, experimental conditions-specific, validation of references. Journal of Experimental Botany. 2009; 60: 487–493.

[21] Gambarotta G, Ronchi G, Friard O, Galletta P, Perroteau I, Geuna S. Identification and validation of suitable housekeeping genes for normalizing quantitative real-time PCR assays in injured peripheral nerves. PLoS ONE. 2014; 9: e105601.

[22] Han X, Xu J, Chen Z, Li P, Zhao L, Tao J, *et al*. Gas5 inhibition promotes the axon regeneration in the adult mammalian nervous system. Experimental Neurology. 2022; 356: 114157.

[23] Kaech S, Banker G. Culturing hippocampal neurons. Nature Protocols. 2006; 1: 2406–2415.

[24] Han Q, Ordaz JD, Liu NK, Richardson Z, Wu W, Xia Y, *et al*. Descending motor circuitry required for NT-3 mediated locomotor recovery after spinal cord injury in mice. Nature Communications. 2019; 10: 5815.
[25] Langnaese K, John R, Schweizer H, Ebmeyer U, Keilhoff G. Selection of reference genes for quantitative real-time PCR in a rat asphyxial cardiac arrest model. BMC Molecular Biology. 2008; 9: 53.
[26] Martínez-Beamonte R, Navarro MA, Larraga A, Strunk M, Barranquero C, Acín S, *et al*. Selection of reference genes for gene expression studies in rats. Journal of Biotechnology. 2011; 151: 325–334.
[27] Peinnequin A, Mouret C, Birot O, Alonso A, Mathieu J, Clarençon D, *et al*. Rat pro-inflammatory cytokine and cytokine related mRNA quantification by real-time polymerase chain reaction using SYBR green. BMC Immunology. 2004; 5: 3.
[28] Seol D, Choe H, Zheng H, Jang K, Ramakrishnan PS, Lim TH, *et al*. Selection of reference genes for normalization of quantitative real-time PCR in organ culture of the rat and rabbit intervertebral disc. BMC Research Notes. 2011; 4: 162.
[29] Bangaru MLY, Park F, Hudmon A, McCallum JB, Hogan QH. Quantification of gene expression after painful nerve injury: validation of optimal reference genes. Journal of Molecular Neuroscience. 2012; 46: 497–504.
[30] Wang Y, Shan Q, Meng Y, Pan J, Yi S. Mrpl10 and Tbp Are Suitable Reference Genes for Peripheral Nerve Crush Injury. International Journal of Molecular Sciences. 2017; 18: 263.
[31] Wang X, Zhao H, Ni J, Pan J, Hua H, Wang Y. Identification of suitable reference genes for gene expression studies in rat skeletal muscle following sciatic nerve crush injury. Molecular Medicine Reports. 2019; 19: 4377–4387.
[32] Liu W, Yu J, Wang YF, Shan QQ, Wang YX. Selection of suitable internal controls for gene expression normalization in rats with spinal cord injury. Neural Regeneration Research. 2022; 17: 1387–1392.
[33] Schlaudraff J, Paul MH, Deller T, Del Turco D. Precise measurement of gene expression changes in mouse brain areas denervated by injury. Scientific Reports. 2022; 12: 22530.
[34] Ma J, Chen J, Gan M, Chen L, Zhao Y, Niu L, *et al*. Comparison of reference gene expression stability in mouse skeletal muscle via five algorithms. PeerJ. 2022; 10: e14221.
[35] Zhang R, Chen S, Wang X, Gu X, Yi S. Cell populations in neonatal rat peripheral nerves identified by single-cell transcriptomics. Glia. 2021; 69: 765–778.
[36] Cesmebasi A. Anatomy of the Dorsal Root Ganglion. In Tubbs RS, Rizk E, Shoja MM, Loukas M, Barbaro N, Spinner RJ (eds.) Nerves and Nerve Injuries, 471–476. 1st Edition. Elsevier. San Diego. 2015.
[37] Mohseni S. Neurologic damage in hypoglycemia. Handbook of Clinical Neurology. 2014; 126: 513–532.
[38] Guo X, Yao Y, Tao Y. Role of DNA Methylation in Chronic Pain. In Bai G, Ren K (eds.) Epigenetics of Chronic Pain, 99–110. 1st Edition. Elsevier, Amsterdam. 2019.
[39] He Z, Jin Y. Intrinsic Control of Axon Regeneration. Neuron. 2016; 90: 437–451.
[40] Vandesompele J, De Preter K, Pattyn F, Poppe B, Van Roy N, De Paepe A, *et al*. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genome Biology. 2002; 3: RESEARCH0034.
[41] Andersen CL, Jensen JL, Ørntoft TF. Normalization of real-time quantitative reverse transcription-PCR data: a model-based variance estimation approach to identify genes suited for normalization, applied to bladder and colon cancer data sets. Cancer Research. 2004; 64: 5245–5250.
[42] Ren G, Juhl M, Peng Q, Fink T, Porsborg SR. Selection and validation of reference genes for qPCR analysis of differentiation and maturation of THP-1 cells into M1 macrophage-like cells. Immunology and Cell Biology. 2022; 100: 822–829.
[43] Xu J, Yang M, Shao AZ, Pan HW, Fan YX, Chen KP. Identification and Validation of Common Reference Genes for Normalization of Esophageal Squamous Cell Carcinoma Gene Expression Profiles. BioMed Research International. 2022; 2022: 9125242.
[44] Stephens AS, Stephens SR, Morrison NA. Internal control genes for quantitative RT-PCR expression analysis in mouse osteoblasts, osteoclasts and macrophages. BMC Research Notes. 2011; 4: 410.
[45] Wisnieski F, Calcagno DQ, Leal MF, dos Santos LC, Gigek CDO, Chen ES, *et al*. Reference genes for quantitative RT-PCR data in gastric tissues and cell lines. World Journal of Gastroenterology. 2013; 19: 7121–7128.
[46] Wu C, Wang X, Zhong M, Liu H, He Q, Yang X, *et al*. Evaluation of potential reference genes for qRT-PCR studies in human hepatoma cell lines treated with TNF-α. Acta Biochimica et Biophysica Sinica. 2013; 45: 780–786.
[47] Yu B, Zhou S, Yi S, Gu X. The regulatory roles of non-coding RNAs in nerve injury and regeneration. Progress in Neurobiology. 2015; 134: 122–139.
[48] Nojima T, Proudfoot NJ. Mechanisms of lncRNA biogenesis as revealed by nascent transcriptomics. Nature Reviews Molecular Cell Biology. 2022; 23: 389–406.