Normalization of Reverse Transcription Quantitative PCR Data During Ageing in Distinct Cerebral Structures

G. Bruckert · D. Vivien · F. Docagne · B. D. Roussel

Abstract Reverse transcription quantitative-polymerase chain reaction (RT-qPCR) has become a routine method in many laboratories. Normalization of data from experimental conditions is critical for data processing and is usually achieved by the use of a single reference gene. Nevertheless, as pointed by the Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines, several reference genes should be used for reliable normalization. Ageing is a physiological process that results in a decline of many expressed genes. Reliable normalization of RT-qPCR data becomes crucial when studying ageing. Here,
we propose a RT-qPCR study from four mouse brain regions (cortex, hippocampus, striatum and cerebellum) at different ages (from 8 weeks to 22 months) in which we studied the expression of nine commonly used reference genes. With the use of two different algorithms, we found that all brain structures need at least two genes for a good normalization step. We propose specific pairs of gene for efficient data normalization in the four brain regions studied. These results underline the importance of reliable reference genes for specific brain regions in ageing.

Keywords RT-qPCR · Ageing · Normalization · geNorm · Normfinder

Introduction

Neurodegenerative diseases represent a real challenge for our societies as their frequencies increase with ageing of the population. These diseases are often associated to gene regulation disorders that make their study useful for the comprehension of the disease. Currently, quantitative PCR is the most efficient tool for this kind of studies because it allows a fast and accurate measurement of gene expression levels. However, the reliability of RT-qPCR is influenced by the selection of reference genes, whose expression must be stable in the experimental conditions studied. However, many studies use "common" reference genes such as *GAPDH* or *actin-beta*, which have been reported to be unstable in certain conditions. Moreover, Sieber and collaborators have demonstrated the importance of using more than one reference gene to strengthen the exactness of measurements when studying ageing or brain insults such as ischaemia. However, the authors focused on the whole brain instead of studying several structures separately.

By using different algorithms, we identified specific combinations of housekeeping genes in the cortex, the hippocampus, the striatum and the cerebellum, allowing a reliable measurement of gene expression during a longitudinal study in RT-qPCR.

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Reverse transcription quantitative-polymerase chain reaction (RT-qPCR) is widely used in gene expression analysis because of its simple, reproducible and high-throughput features. It gives a rapid means of understanding gene variation in various organisms/organs by measuring the expression of target genes compared to reference genes across different samples. On top of that, RT-qPCR has become a low-cost method allowing a generalization of its use in laboratories. However, to analyse RT-qPCR data, several factors such as sample amount, RNA integrity or complementary DNA (cDNA) quality must be controlled because they affect the quantitative measurement of gene expression [1, 2]. Thus, in order to obtain a robust analysis of gene expression, reference genes are used as an internal control to normalize and monitor the variations of expression between samples and reactions [3, 2, 4]. The expression of these reference genes should not vary in the tissues or cells under investigation or in response to experimental treatment [3]. As shown by two surveys realized in 2013, there are still many bias in RT-qPCR studies: the

stability of reference genes is not systematically checked, and normalization is generally achieved by the use of a single reference gene [2], which is in discordance with the Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines published by Bustin and collaborators to avoid misinterpretations of RT-qPCR results [5].

Ageing is a physiological process associated with deficits in learning and memory tasks, explained by deficits in key brain functions, like motor coordination [6] and sensory and cognitive functions [7]. Ageing is also a risk factor for many neurological diseases, including cerebral ischaemia [8], Alzheimer's disease [9], Parkinson's disease [10] and others. To study gene expression during ageing in neurodegenerative pathologies or in normal brain, RT-qPCR has been widely used. It has been suggested that RT-qPCR data should be normalized to at least two reference genes whose expression has been demonstrated to be stable in the conditions studied [3, 11]. The most frequently used genes for normalization are glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) and beta-actin (*ActinB*). However, they can display variable expressions in many paradigms. For example, *ActinB* has been shown to vary in several studies, like in ischaemic/hypoxic conditions [12, 13], and *GAPDH* is not stable in a model of apoptosis induced by age on neurones [14]. Also, their sequences overlap with highly homologous segmental duplications sequences [15].

Based on that, Sieber and collaborators published a useful study in 2010 that demonstrated the need to check the stability of normalization genes in ageing and ischaemia for brain studies [16]. However, the authors did not discriminate different structures/areas but rather used the whole brain. It is well known that neurodegenerative diseases/models affect specific brain regions, and gene expression also varies in different structures. By using the *geNorm* and the *Normfinder* methods, we studied the stability of common reference genes, such as beta-actin (*ActinB*), polymerase (RNA) II (DNA directed) polypeptide A (*Polr2a*), succinate dehydrogenase complex, subunit A (*Sdha*), protoporphyrinogen oxidase (*Ppox*), hypoxanthine guanine phosphoribosyl transferase (*Hprt*), ribosomal protein L13a (*Rpl13a*), hydroxymethylbilane synthase (*Hmbs*), peptidylprolyl isomerase B, or cyclophilin B,(*Ppib*) and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) in four mouse brain structures (cortex, hippocampus, striatum and cerebellum) during ageing (Figs. 1, 2, 3 and 4). We found that the stability of each gene depends on the studied structure and that reference genes are specific for each structure in ageing. We thus propose, for each of these brain structures, a set of reference genes usable for ageing studies in mice, and we verified our reference genes by measuring the expression of the age-related gene tissue plasminogen activator (tPA) in the hippocampus during ageing.

Results

RT-qPCR Efficiency

Quantification cycle (*Cq*) was correlated to the starting concentration of the dilution series (from 1:10 to 1:10, 000; five orders of magnitude). The coefficient of correlation (R^2) ranged from 0.977 to 0.998 in the cortex, 0.971 to 0.986 in the hippocampus, 0.977 to 0.993 in the striatum and 0.975 to 0.994 in the cerebellum. The efficiency was then calculated as described in the "Material and Methods" and ranged from 82.45 to 126.625 % as shown in the Table 1. Non-normalized Cq values in our experiments were all within the range of the dilution series.

Expression of Reference Genes in the Brain

We measured the raw expression (here called starting quantity) of our nine genes in four cerebral structures: the cortex, hippocampus, striatum and cerebellum. Among the tested genes, we observed variations during ageing in the different brain structures. For example, in the cortex, *hmbs*, *Actinb*, *Ppox*, *Polr2a* and *GAPDH* do not vary between 8 weeks, 7 months, 17 months and 22 months in contrast to *Ppib*, *Rpl13a*, *Hprt* and *Sdha* (Table 2). In the hippocampus, only *Ppib*, *Hprt* and *Polr2a* show no statistical differences of expression during ageing (Table 2). In the striatum, *Ppib*, *Rpl13a*, *Actinb*, *Ppox*, *Sdha*, *Polr2a* and *GAPDH* do not show statistical differences of expression during ageing, whereas in the cerebellum, only *Ppib*, *Rpl13a* and *GAPDH* show no statistical differences (Table 2). An intragroup analysis (Mann and Whitney test) was also performed on the raw data expression (Supplementary table 2).

These results clearly demonstrate variations in many commonly used reference genes, but also differences between brain structures during normal ageing. It also demonstrates that one reference gene is usually not enough, and normalization to multiple reference genes needs to be done for each cerebral structure during ageing. To address this, we used two different methods: by using the qBase+™ software that contains the geNorm algorithm and by using the Normfinder algorithm.

Determination of the Correct Reference Genes in Cerebral Structures

Cortex

In the cerebral cortex, multiple analyses of the nine reference genes revealed a geNorm *V* value under the 0.15 threshold for the use of two genes including *Actb*, *Polr2a*, *Sdha*, *Ppox*, *Hprt*, *Rpl13a*, *Hmbs*, *Ppib* and *GAPDH*
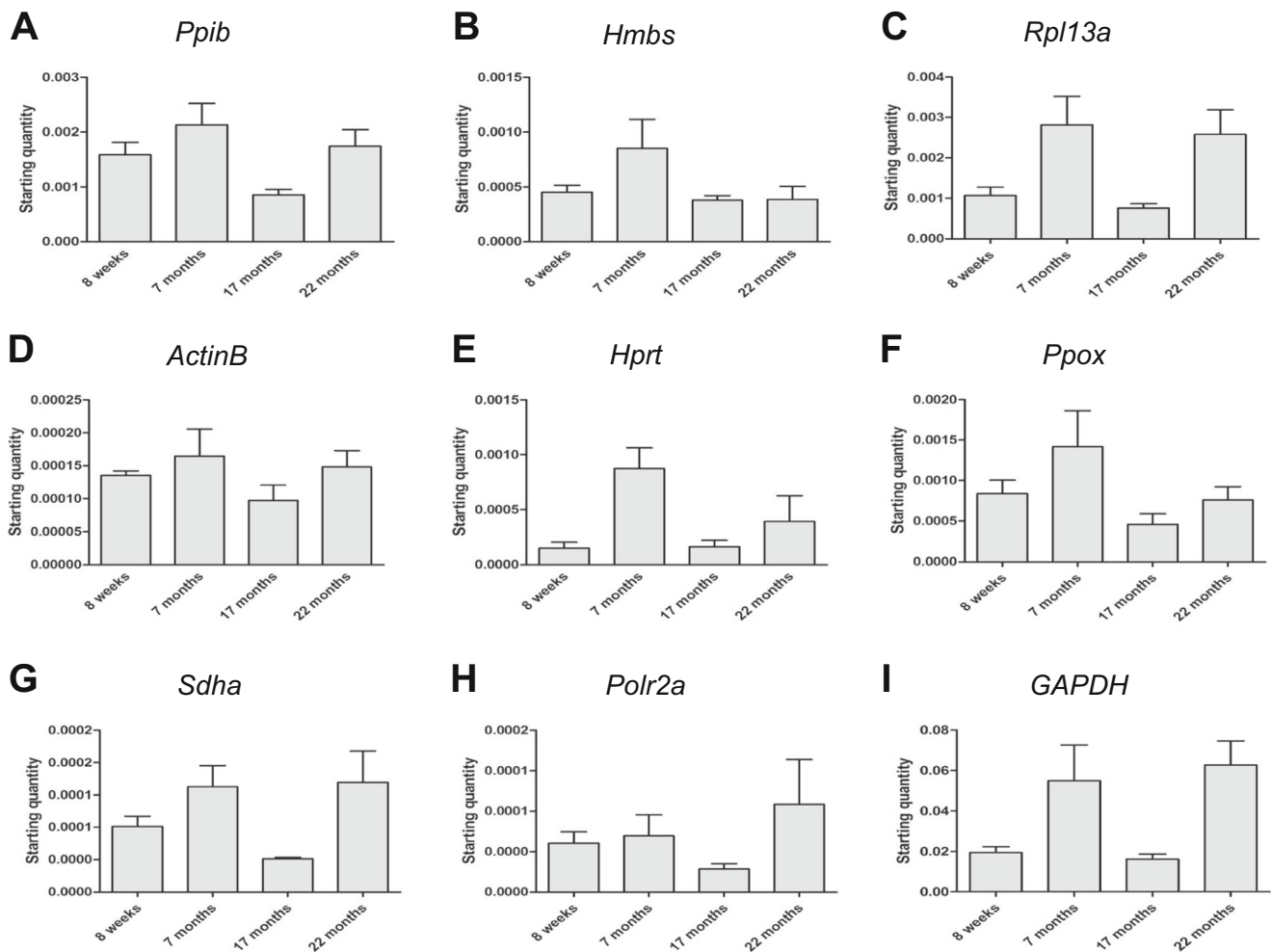
Fig. 1 Raw data of RT-qPCRs performed on the cortex of mice aged from 8 weeks to 22 months old. Starting quantity was directly obtained from the Cq value using the Bio-Rad CFX Manager software for *Ppib* (**a**), *Hmbs* (**b**), *Rpl13a* (**c**), *ActinB* (**d**), *Hprt* (**e**), *Ppox* (**f**), *Sdha* (**g**), *Polr2a* (**h**) and *GAPDH* (**i**). $n=5$ independent animals in each group

(Fig. 5a). The geNorm *M* value [17] showed that the two best genes to use as reference genes in the cerebral cortex during ageing are *Rpl13a* and *GAPDH* (Fig. 5b): between 8 weeks and 22 months, these two genes display the same profile of expression in all our studied conditions.

To ensure the choice of the reference genes in the cortex, we also performed a Normfinder analysis that calculates a stability value after the estimation of an intra- and intergroup variation (see "Material and Methods" [1]). The ranking of the reference genes (Fig. 5c) showed that the best genes for normalizing RT-qPCR data in the cortex during ageing are *Rpl13a* and *GAPDH*.

Hippocampus

The same method was applied in the hippocampus, aged from 8 weeks to 22 months old. Again, among the nine selected genes, two paired genes were enough to normalize RT-qPCR data in the hippocampus during ageing, as shown by the geNorm *V* value (Fig. 6a). According to the geNorm *M* value, *Rpl13a* and *Hprt* is the best pair of reference genes to normalize RT-qPCR data in the hippocampus during ageing (Fig. 6b).

The Normfinder analysis gave *Rpl13a* and *Sdha* as the most stable genes (Fig. 6c). There was a slight difference in the choice of genes between geNorm and Normfinder. However, both analyses ranked *Rpl13a* as the most stable gene in the hippocampus during ageing.

Striatum

In the striatum of C57BL6/J mice aged from 8 weeks to 22 months old, analysis of the studied reference genes revealed that two genes of the list are enough to normalize RT-qPCR data (Fig. 7a). The geNorm *M* value showed that *Rpl13a* and *Ppib* are the best candidates for the normalization of RT-qPCR data in the striatum during ageing (Fig. 7b), and the Normfinder analysis (Fig. 7c) gave the same result.

Cerebellum

Regarding the cerebellum in ageing, the analysis of the geNorm *V* value showed that two reference genes are also enough to normalize RT-qPCR (Fig. 8a). According to the geNorm *M* value, these genes are *GAPDH* and *pPIB* (Fig. 8b). The Normfinder analysis gave a slightly different result, as the two best genes were *GAPDH* and *Hprt* (Fig. 8c).

Evaluation of Reference Genes in RT-qPCR Experiments

To test our hypothesis, we decided to check the expression of a gene during ageing. As it was previously demonstrated that tissue plasminogen activator (tPA) was impacted during ageing [18–20], we decided to study its expression in the hippocampus of mice from 8 weeks to 22 months old.

By using *Rpl13a* and *Hprt*, the two recommended reference genes of the geNorm analysis, we observed that the expression of *tPA* was decreased during ageing (Fig. 9a, $p=$ 0.0079; Kendall's correlation test). However, when using

Hmbs and *ActinB*, commonly considered as "standard" reference genes, and also under the threshold of 0.5 on the geNorm *M* value, no changes in the expression of tPA were observed during ageing (Fig. 9b, $p=0.7399$; Kendall's correlation test). These results highlight the importance of efficient normalization for reliable results in gene expression studies in ageing.

Discussion

RT-qPCR is a useful and sensitive technique that gives a large amount of data within a short amount of time and is able to detect small differences of expression. However, because of technical variations (storage condition, RNA isolation and purification, amount of starting material, enzymatic efficiency…), normalization is an obligatory step [11, 21, 4]. Since RT-qPCR has become a routine technique for molecular biology laboratories, numbers of reference genes have been used, with glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*) or beta-actin (*ActinB*) as gold standards. However, in many
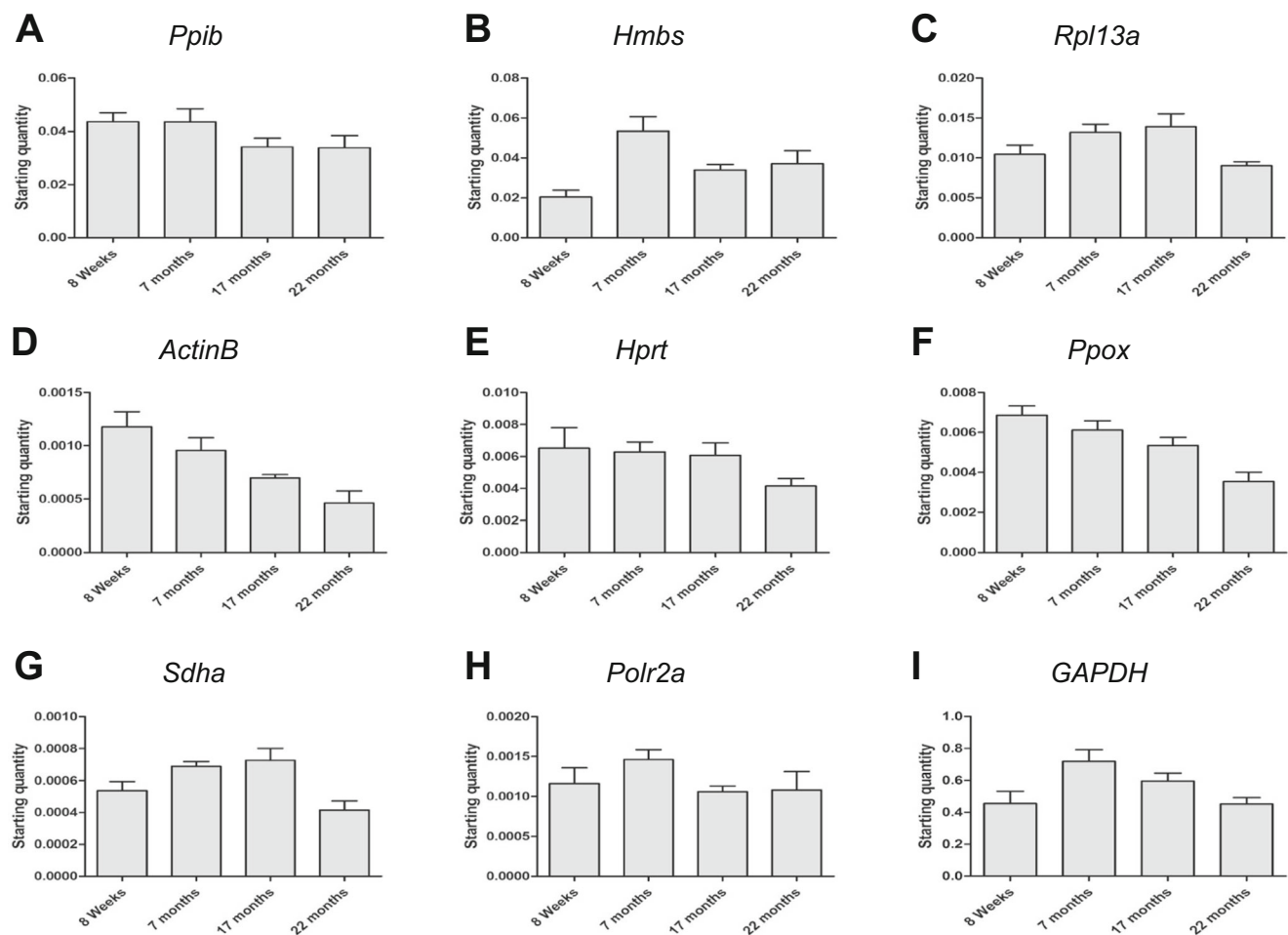

Fig. 2 Raw data of RT-qPCRs performed on the hippocampi of mice aged from 8 weeks to 22 months old. Starting quantity was directly obtained from the Cq value using the Bio-Rad CFX Manager software for *Ppib* (**a**), *Hmbs* (**b**), *Rpl13a* (**c**), *ActinB* (**d**), *Hprt* (**e**), *Ppox* (**f**), *Sdha* (**g**), *Polr2a* (**h**) and *GAPDH* (**i**). $n=5$ independent animals in each group
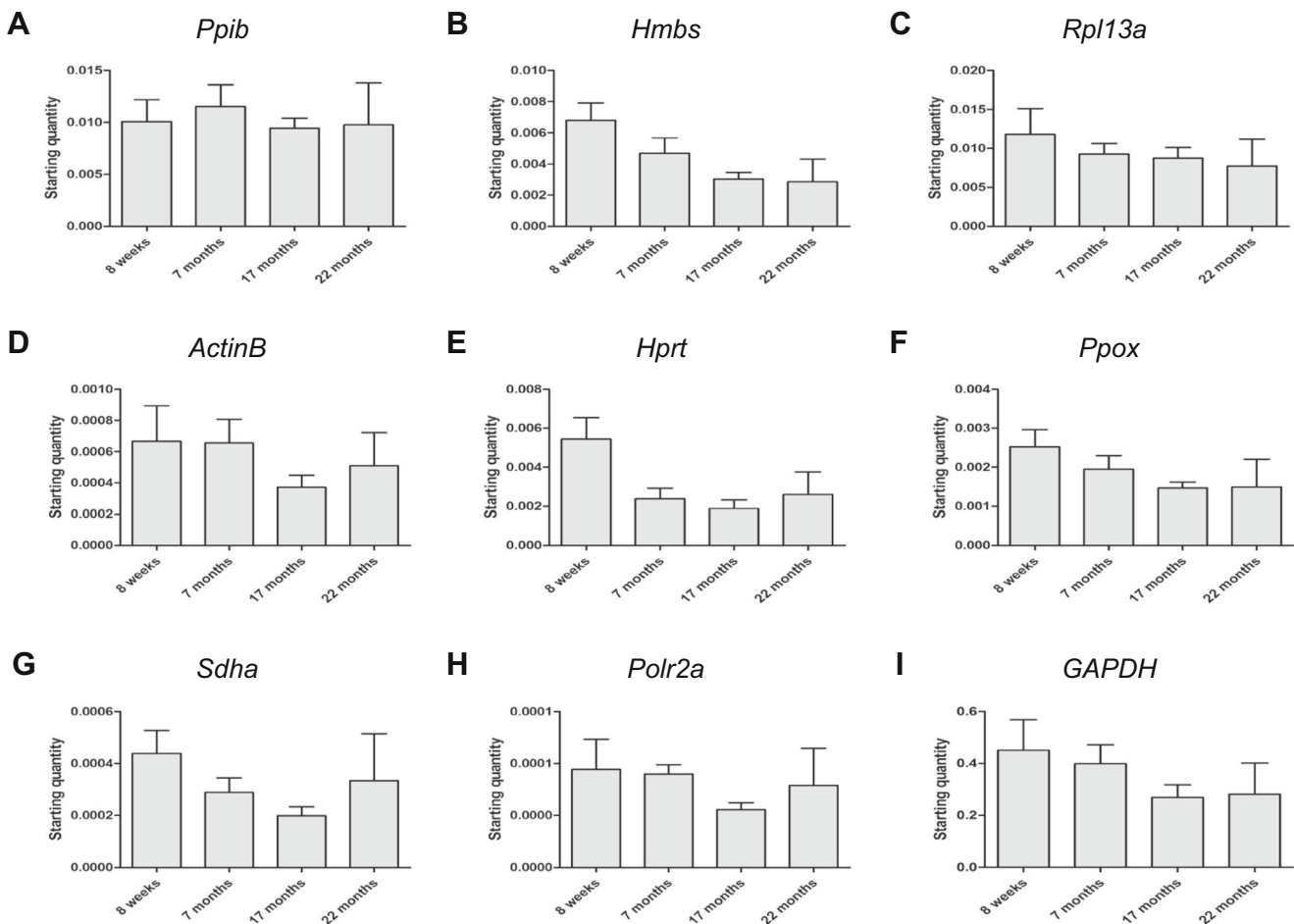
Fig. 3 Raw data of RT-qPCRs performed on the striatum of mice aged from 8 weeks to 22 months old. Starting quantity was directly obtained from the Cq value using the Bio-Rad CFX Manager software for *Ppib* (a), *Hmbs* (b), *Rpl13a* (c), *ActinB* (d), *Hprt* (e), *Ppox* (F), *Sdha* (G), *Polr2a* (H) and *GAPDH* (i). *n*=5 independent animals in each group

cases, these genes have been demonstrated to be unstable, like *ActinB* during cerebral ischaemia [12], or in many other conditions (reviewed in [22]).

In the present study, we show that (i) during ageing, in different cerebral structures, there are variations of expression for nine commonly used reference genes (*Actinb*, *Polr2a*, *Sdha*, *Ppox*, *Hprt*, *Rpl13a*, *Hmbs*, *Ppib* and *GAPDH*), (ii) at least two genes are necessary for a good normalization process in brain structures during ageing and (iii) each cerebral structure studied here (cortex, hippocampus, striatum and cerebellum) requires specific paired-reference genes (Table 3).

It has already been suggested by Sieber and colleagues in 2010 that in physiological brain during ageing, RT-qPCR needs to be normalized to more than one reference gene [16]. They performed the study on whole brains during physiological ageing (from 8 weeks to 24 months) but also in animals subjected to a model of stroke. It appeared that ischaemia leads to the modification of the reference genes to use for normalization of RT-qPCR data in ageing (*Hprt* and *Hmbs* vs *ActinB* and *Hmbs*).

Despite the importance of reference genes, especially for ageing, many studies use only one reference gene and do not check its stability in experimental conditions as recommended by the MIQE [5]. Here, we show the importance of using at least two reference genes to normalize RT-qPCR data, but also the importance of checking their stability of expression. It is confirmed by studying the expression of tPA during ageing in the hippocampus. By using the geNorm-recommended reference genes, we observed a decrease in tPA expression during ageing, as previously reported [18–20]. However, when we used two other genes commonly considered as "standard" reference genes, we did not see any difference in tPA expression with age.

It is interesting to note that for the hippocampus and the cerebellum, there is a small difference between the two proposed reference genes. Indeed, for the hippocampus, for example, the geNorm analysis recommends *Rpl13a* and *Hprt*, while the Normfinder analysis recommends *Rpl13a* and *Sdha*. This is probably due to the difference between the two algorithms: geNorm gives a stability value on the principle that the expression ratio of two ideal internal control genes is identical in all samples [3]; whereas Normfinder combines the intra- and the
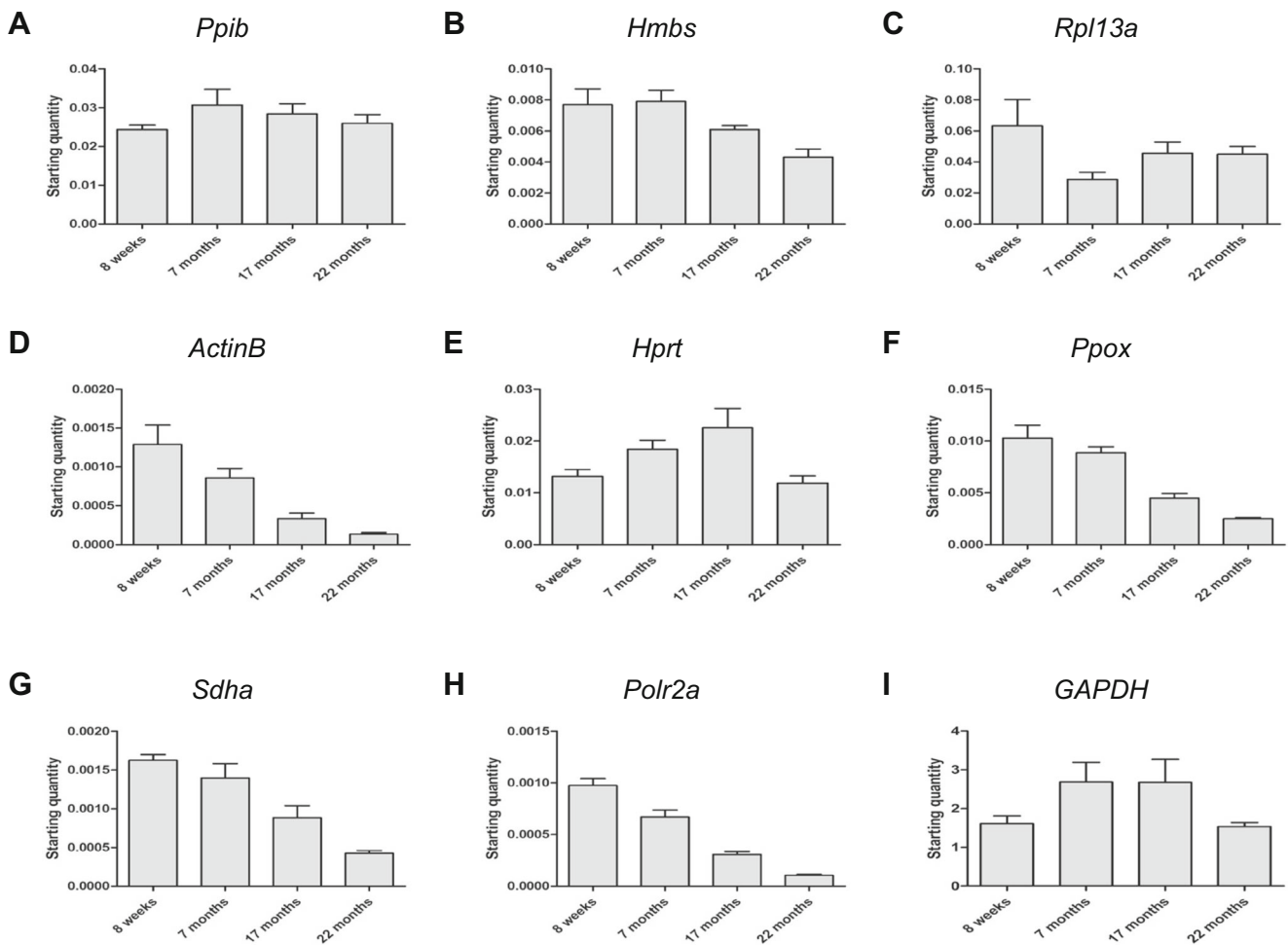
Fig. 4 Raw data of RT-qPCRs performed on the cerebellum of mice aged from 8 weeks to 22 months old. Starting quantity was directly obtained from the Cq value using the Bio-Rad CFX Manager software for *Ppib* (**a**), *Hmbs* (**b**), *Rpl13a* (**c**), *ActinB* (**d**), *Hprt* (**e**), *Ppox* (**f**), *Sdha* (**g**), *Polr2a* (**h**) and *GAPDH* (**i**). $n=5$ independent animals in each group

intergroup variation into a stability value [1]. However, when there is a difference between the two methods, the reference genes are always close to the most stable genes, reflecting their ability to be a good reference gene.

Our data also reveal that each structure needs its own reference paired genes. This is very important as many neurological diseases are structure specific. In stroke, depending of the model used, the majority of the infarct lesion is in the cortex

Table 1 List of the nine reference genes used and their forward and reverse primers sequences. Ensembl (CCDS) numbers, length of the RT-qPCR transcripts, and efficiency of each primers are also indicated gene

Name	Forward	Reverse	CCDS number	Transcripts length (base pairs)	Primer efficiency (%)
Actb	gcagctccttcgttgccggt	tacagcccggggagcatcgt	CCDS19833	133	99,175
Polr2a	gaaggttttgcgctgtgtct	tggccaggatgtccttaatc	CCDS70217	83	108,925
Sdha	tcatcggtgttgctgtgtg	cactccccattttccatcag	CCDS26643	135	104,85
Ppox	ggcaaagagcctgatgaaac	gcaaatactcctcggcaaag	CCDS15487	101	82,45
Hprt	ctttgctgacctgctggatt	tatgtccccgttgactgat	CCDS40972	121	103,15
Rpl13a	tacgctgtgaaggcatcaac	gggaggggttggtattcatc	CCDS21230	96	102,875
Hmbs	gaaatcattgctatgtccacca	gcgttttctagctccttggtaa	CCDS23106	98	100,75
Ppib	cagcaagttccatcgtgtca	gatgctctttcctcctgtgc	CCDS23301	85	108,175
GAPDH	tgcgacttcaacagcaactc	atgtaggcaatgaggtccac	CCDS51913	143	126,625

Table 2 Kruskal-Wallis test of the nine genes in the four studied cerebral structures during ageing. The test was performed on non-normalized starting quantities with Graph Prism software

	Cortex	Hippocampus	Striatum	Cerebellum
Ppib	0.0407*	0.2252	0.7391	0.5919
Hmbs	0.5114	0.0078**	0.0344*	0.0047**
Rpl13a	0.0221*	0.0047**	0.6558	0.3949
ActinB	0.4707	0.0011**	0.4552	<0.0001***
Hprt	0.0236 *	0.0994	0.0263*	0.0328*
Ppox	0.3813	0.0006***	0.25	<0.0001***
Sdha	0.0017**	0.0045**	0.1322	<0.0001***
Polr2a	0.2680	0.1894	0.2904	<0.0001***
GAPDH	0.0501	0.0279*	0.5062	0.0593

*p<0.05; **p<0.01; ***p<0.001

and in the striatum [23]; various models of Parkinson's disease affect the substantia nigra [24]; in models of Alzheimer's disease, the first amyloid deposits are structure specific depending of the model used [25]. Moreover, a cofactor of all of these diseases is ageing, and it has been previously shown that ageing plays important roles in stroke [18] for example.

This is particularly important for pathological studies in ageing, as the pathology by itself will change the reference genes needed for a good normalization process [16]; however, the same attention should be used for cerebral structure selection, instead of working on whole brain, as we demonstrated here.

Our study highlights the importance of the brain structure studied, by showing that each structure needs its own specific reference genes. It also shows that a stability expression control is primary, as demonstrated by the expression of tPA within the hippocampus. It comes in addition with the MIQE guidelines [5] and other publications which appeal for "good practices" in RT-qPCR studies [2, 1, 3].

Age is a common factor to many neurological disorders, and RT-qPCR made from brain tissue in ageing should be well designed to avoid misinterpretations of the data. Sieber and colleagues were the first ones to underline this specific point in neurological disorders [16]. However, they performed their study on the whole brain. Many neurological studies are more focused on a specific cerebral area (hippocampus, cortex, striatum…) depending on the neurological disease studied. Here, we demonstrate that each cerebral structure needs its own reference genes validation, and we propose the best couple of genes for the cortex, the hippocampus, the striatum and the cerebellum for ageing studies in the C57BL6/J mice. However, for pathological studies in ageing, the stability expression of reference genes needs to be checked.
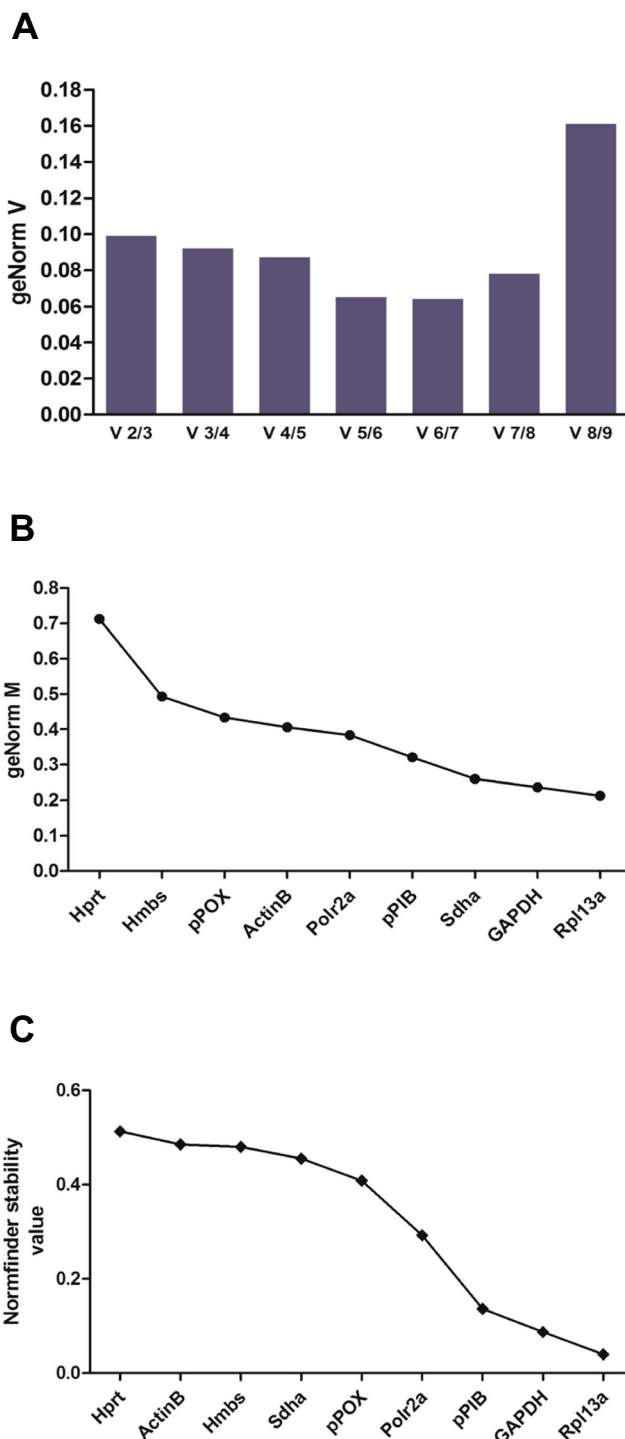
Fig. 5 Determination of the best reference genes in the cortex during ageing using the geNorm *M* algorithm. **a** Two reference genes are enough in the cortex to normalize RT-qPCR data as the pairwise variation *V* value for *V* 2/3 (*n/n*+1) is under 0.15. **b** Best genes to study ageing in the cortex are *Rpl13a* and *GAPDH* according to the geNorm *M* value under 0.5 for these genes. **c** The use of the Normfinder algorithm also ranked *Rpl13a* and *GAPDH* as the best reference genes for normalizing RT-qPCR data in the cortex during ageing
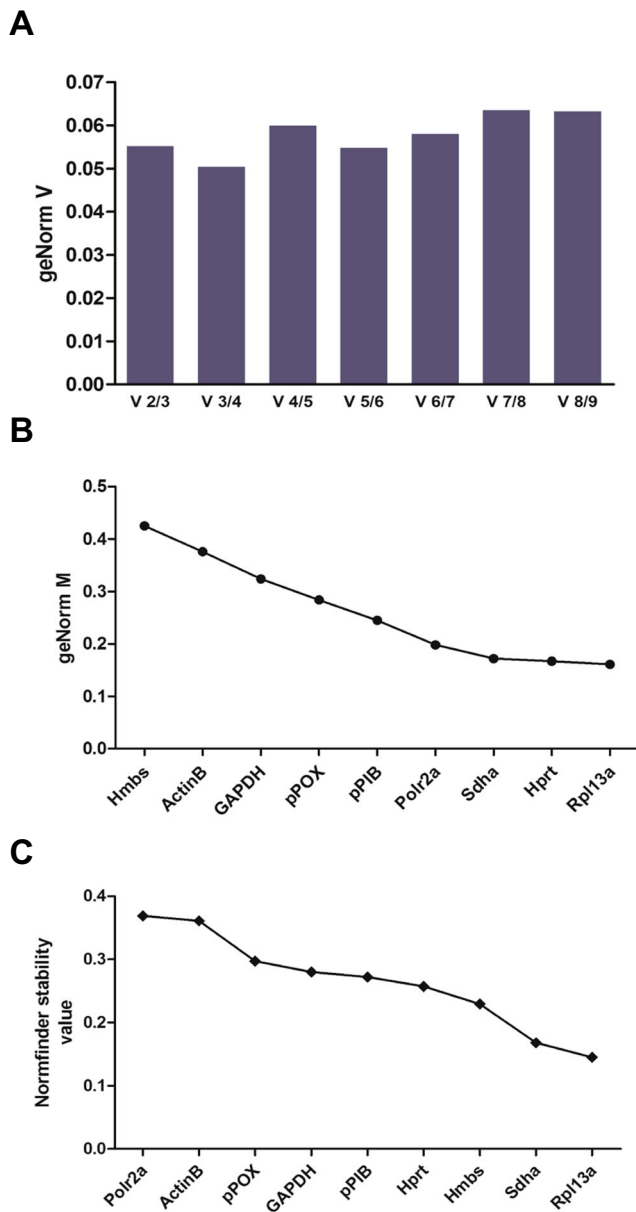
A

B

C

Fig. 6 Determination of the best reference genes in the hippocampus during ageing using the geNorm M algorithm: **a** Two reference genes are enough in the hippocampus to normalize RT-qPCR data as the pairwise variation V value for V 2/3 ($n/n+1$) is under 0.15. **b** Best genes to study ageing in the hippocampus are *Rpl13a* and *Hprt* according to the geNorm M value under 0.5 for these genes. **c** The use of the Normfinder algorithm ranked *Rpl13a* and *Sdha* as the best reference genes for normalizing RT-qPCR data in the hippocampus during ageing

Material and Methods

Aged Animals

Our study was performed on male C57BL/6 mice of distinct age and approved by local (French Legislation act no. 87–848, Ministère de l'Agriculture et de la Forêt) and European

(directives from the European Communities Council, November 24, 1986, 86/609/EEC) authorities. C57BL/6 mice have a lifespan of ~26 months [26]. Four groups were designed: 8 weeks old, 7 months old, 17 months old and 22 months old, with five mice per group. Animals were housed in a temperature-controlled room on a 12-h-light/12-h-dark cycle, with food and water given ad libitum.
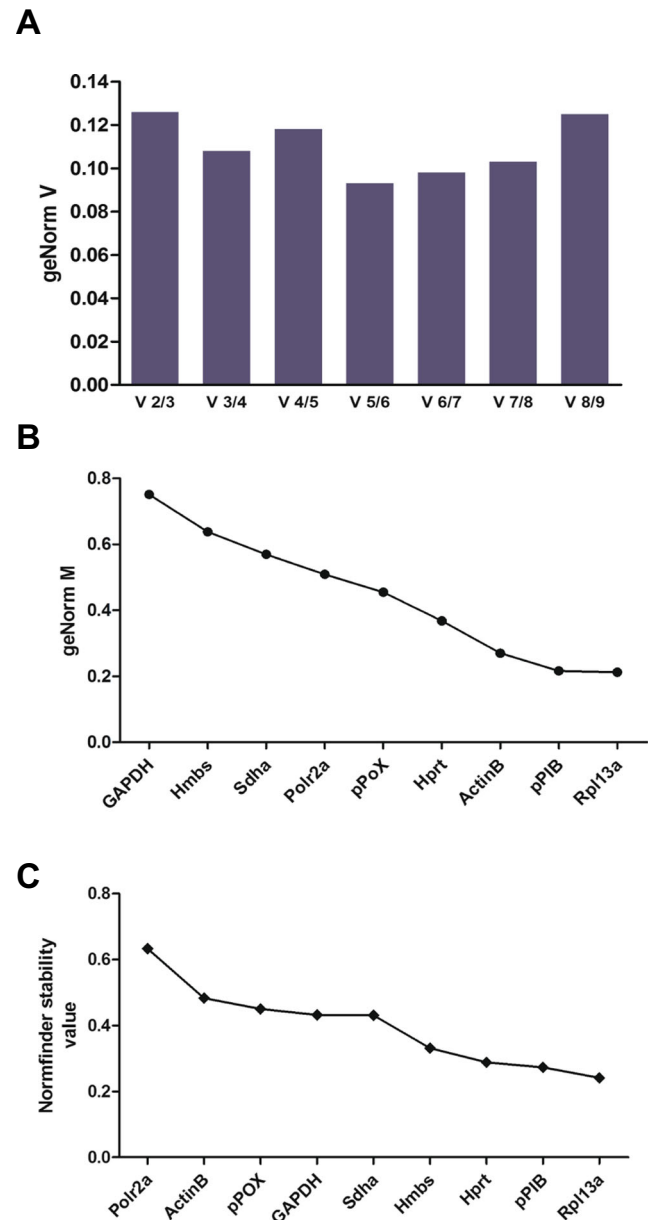
A

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C

Fig. 7 Determination of the best reference genes in the striatum during ageing using the geNorm M algorithm. **a** Two reference genes are enough in the striatum to normalize RT-qPCR data as the pairwise variation V value for V 2/3 ($n/n+1$) is under 0.15. **b** The best genes to study ageing in the striatum are *Rpl13a* and *Ppib* according to the geNorm M value under 0.5 for these genes. **c** The use of the Normfinder algorithm also ranked *Rpl13a* and *Ppib* as the best reference genes for normalizing RT-qPCR data in the cortex during ageing
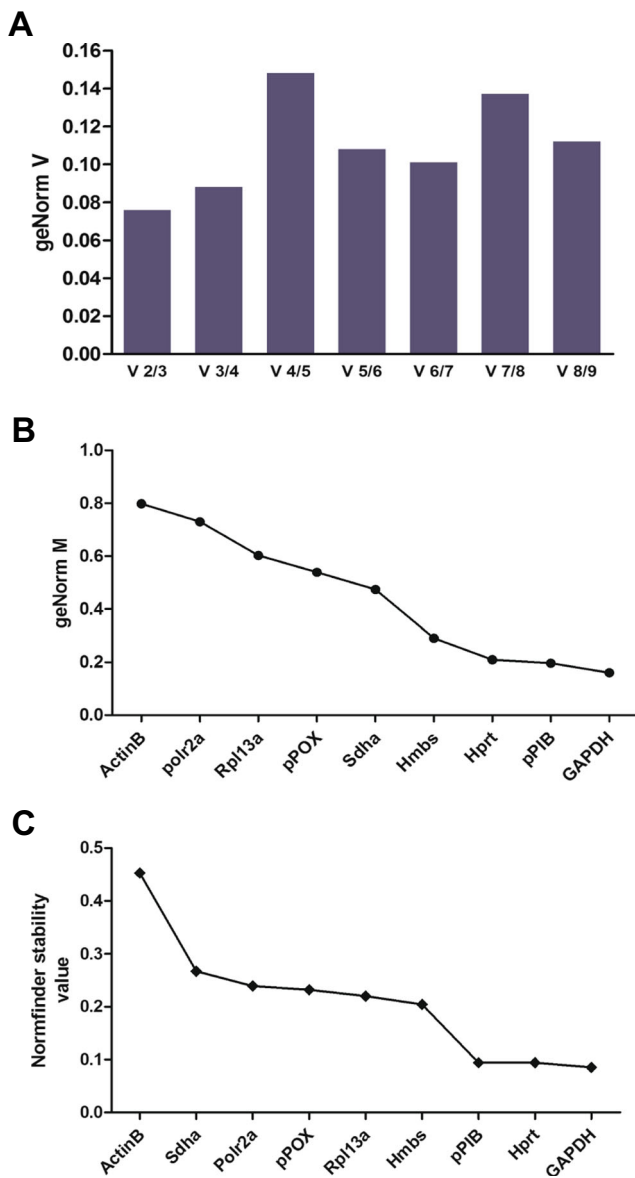
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Fig. 8 Determination of the best reference genes in the cerebellum during ageing using the geNorm *M* algorithm: **a** Two reference genes are enough in the cerebellum to normalize RT-qPCR data as the pairwise variation *V* value for *V* 2/3 (*n/n*+1) is under 0.15. **b** The best genes to study ageing in the cerebellum are *GAPDH* and *Ppib* according to the geNorm *M* value under 0.5 for these genes. **c** The use of the Normfinder algorithm ranked *GAPDH* and *Hprt* as the best reference genes for normalizing RT-qPCR data in the cortex during ageing

Brain Sample Preparation

Mice were anaesthetized with 5 % isoflurane in a 70/30 % mixture of NO_2/O_2 and perfused with cold heparinized NaCl 0.9 % to wash out blood cells. Brains were excised and precisely dissected under a binocular microscope to isolate the cerebral cortex, hippocampus, cerebellum and striatum. Tissue sections were maintained at −80 °C until messenger RNA (mRNA) extraction.
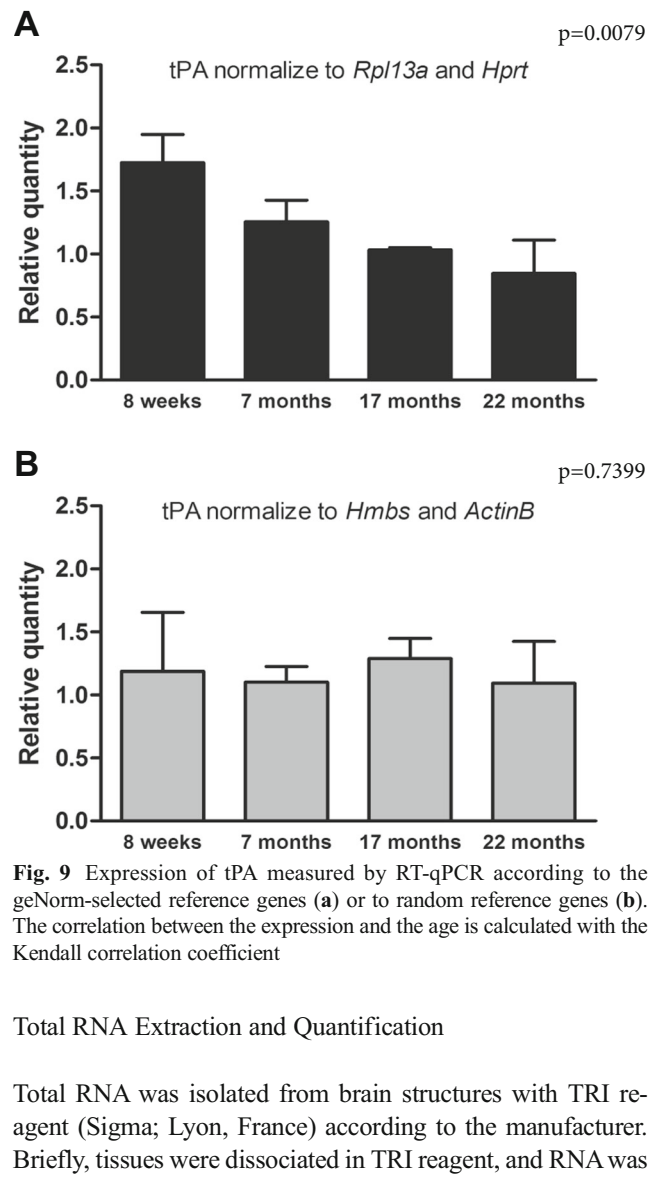
A

B

Fig. 9 Expression of tPA measured by RT-qPCR according to the geNorm-selected reference genes (**a**) or to random reference genes (**b**). The correlation between the expression and the age is calculated with the Kendall correlation coefficient

Total RNA Extraction and Quantification

Total RNA was isolated from brain structures with TRI reagent (Sigma; Lyon, France) according to the manufacturer. Briefly, tissues were dissociated in TRI reagent, and RNA was isolated by the addition of chloroform. Total RNA was washed by ethanol and treated with TURBO DNase (Ambion; Saint Aubin, France) to avoid DNA contamination. Total RNA was quantified by spectrophotometry (NanoDrop Technologies; Wilmington, USA).

Reverse Transcription

We performed the reverse transcription from 1 µg of total RNA and the iScript kit (iScript Select cDNA Synthesis Kit,

Table 3 Choice of reference genes for RT-qPCR normalization after geNorm analysis

Structures	Housekeeping genes	
Cortex	*Rpl13a*	*GAPDH*
Hippocampus	*Rpl13a*	*Hprt*
Striatum	*Rpl13a*	*Ppib*
Cerebellum	*GAPDH*	*pPIB*

Bio-Rad; Marnes-la-Coquette, France) in a total volume of 20 μL with the following cycle conditions: 42 °C (90 min); 85 °C (5 min). The cDNA products were then stored at −20 °C until their use.

Quantitative PCR

RT-qPCR were performed from 1 μL of 1:20 diluted cDNA in 15 μL total of a 1× solution of iQ SYBR Green Supermix (Bio-Rad; Marnes-la-Coquette, France) containing 200 nM of each primer. Based on mRNA coding sequences (www.ensembl.org), mouse-specific primers were designed by using the Primer3Plus software (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi) [27] (sequences are in Table 1). Assays were run in triplicate on the CFX96 real-time system c1000 thermal cycler (Bio-Rad; Marnes-la-Coquette, France), with the following cycle conditions: 95 °C (3 min); [95 °C (2 s), 60 °C (20 s)]×39; 70 °C (30 s).

Data Analysis

Each plate contains the whole samples (triplicate of the 5 mice per group) for one gene and one cerebral structure (8 genes, 4 structures: 32 plates of RT-qPCR), and Cq values were obtained from the Bio-Rad CFX Manager software (Bio-Rad; Marnes-la-Coquette, France). Relative gene expression values (starting quantity) were calculated with the Bio-Rad CFX Manager software and corrected with each efficiency (E) inferred from 5-fold standard dilution series, as described by Pfaffl [28].

$$E = 10 - (1/\text{slope})$$

Kruskal-Wallis test was performed on the starting quantities to check the stability of each gene in the studied structures.

To investigate the best normalization process to reference genes, we used the geNorm algorithm associated to qBase+™ software and the Normfinder algorithm. geNorm allows the calculation of stability (M value) and the ranking of selected reference genes, on the basis of non-normalized expression levels [3, 17]. It also suggests the number of internal controls to use in the selected experimental conditions by giving the V value. It is stated that the benefit of using an extra ($n+1$) reference gene is limited as soon as the V $n/n+1$ value drops below the 0.15 threshold. Calculations are detailed as follows [3]:

The qBase+ software calculates relative quantities (RQs) for each gene in each sample by comparing the Cq of a given sample with the average Cq across all samples for that gene,

taking into account differences in PCR amplification efficiencies (E) (Eq. 1).

$$\Delta\text{Cq} = \frac{\sum_{i=1}^{n}\text{Cq}_i}{n} - \text{Cq}_{\text{sampleA}} = \text{Cq} - \text{Cq}_{\text{sampleA}} \tag{1}$$

with n = number of samples

$$\text{RQ}_A = E^{\Delta\text{Cq}}$$

Then, the RQ is normalized by dividing it by the geometric mean Cq of a set of selected reference genes, which results in the normalized relative quantity (NRQ). The reference genes are measured in all samples of the experiment. In theory (if there was no variability), each reference gene should have the same RQ values in all samples. In reality, factors are responsible for variations in the expression levels of the reference genes (amount of cDNA, RNA integrity, enzyme efficiency, …). However, this variation is a direct measure of the noise between the samples and can be used to calculate normalization factors (NF=factor to be multiplied to the observed RQ values so that the expression levels of the housekeeping genes are equalized across samples) for each sample. For NRF, a variation of 2- to 4-fold is generally accepted and based on the geometric mean as shown in Eq. 2.

$$\text{NF} = \frac{1}{\text{geometric mean (RQ}_{\text{refs}})} \tag{2}$$

Geometric mean of n values $= \sqrt[n]{a_1 a_2 \cdots a_n}$.

These normalization factors are then used to adjust the RQ values of the genes accordingly so that the variability is eliminated. Here, we used nine housekeeping genes in our study, and NF varies from 0.398+/−0.031 to 0.946+/−0.103 in our four groups.

Normfinder estimates first variations within the group and then variations between different groups. Having estimated both the intra- and intergroup variation, the algorithm combines the two into a stability value, which adds the two sources of variation and thus represents a practical measure of the systematic error that will be introduced when using the investigated genes [1].

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