Establishing a canine brain and tissue bank – molecular validation by RT-qPCR targeting three reference genes

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Abstract

Background: Dogs (Canis familiaris) are natural models of several human diseases, including age-related dementia. However, the molecular techniques, which are routinely applied in invertebrate and rodent models to study disease pathologies and mechanisms, has limited applicability in dogs, mainly because of ethical reasons. In the case of humans, the limited accessibility of tissue samples is at least partly solved by biobanks, which collect and store tissues and organs from voluntary donations. A similar approach with pet dogs could support both translational and veterinary research goals by providing access to good quality biological materials obtained from a wide range of dogs with known ancestry, life history and medical background. Therefore, we have established an initiative, the Canine Brain and Tissue Bank, to collect and store biological samples from pet dogs.

Objectives: The molecular qualities of tissue samples collected and stored on a tissue bank are crucial for reliable downstream applications. We used quantitative Real-Time PCR methodology to assess the stability of mRNA content in our stored samples. Three previously validated reference genes, GAPDH , HMBS and HPRT1 were chosen as targets.

Results: The tested reference genes showed expression patterns and stability values that were consistent with the literature.

Conclusions: Based on the results, the molecular quality of tissues collected in the CBTB's donations system can fit in the standards of dog gene expression analyses.

Background

Dogs (*Canis familiaris)* have been proposed as natural models of several human diseases, including age-related pathologies [1–5], and offer many advantages over canonical laboratory model organisms in regard to translatability [6, 7]. Laboratory dogs have already been utilized to study various aspects of cognitive aging [8, 9]. However, recently pet dogs have been proposed as even more valuable models in this field to study aging, dementia and possible interventions [6, 7, 10, 11].

The main reasons for pet dogs to outdo laboratory dogs in dementia research lie in their greater natural variability, including a wide range of expected lifespans across breeds [12, 13]. In addition, the variable living environment and nutritional background of these companion animals better corresponds with people. On the other hand, laboratory dogs, under well established and approved protocols, can be sacrificed to obtain valuable information about ultrastructural, intracellular and molecular changes in their brains. Apparently, this approach cannot be applied in the case of pet dogs, unless their euthanasia is justified by serious medical reasons unlinked to research purposes. A few studies [14, 15] have used brain tissues from pet dogs for research purposes, however, in general, the sample size they could obtain was reported to be limited [15].

In human medical research, tissue banks, which accept voluntary donations, and follow strict quality-control protocols, have been introduced to attenuate this problem [16]. Those tissue banks, which focus on collecting brain samples, could be especially relevant for dementia research. Systematic brain banking has been known since 1960 [17] and brain banks across the world have since been organized into collaborative groups, like the BrainNet Europe [18]. Materials provided by these institutions have already led to crucial findings regarding the pathology and characteristic molecular changes of several diseases, including dementia [19, 20].

A canine tissue bank, following human examples, may help overcome the challenges of obtaining organs and tissue specimens from a wide range of pet dogs. In addition, as improving animal welfare standards demand reduction of laboratory animal sacrifices [21, 22], a tissue bank could also provide a good alternative for several research goals as a source of canine tissues. Furthermore, it can also serve veterinary research and educational purposes in many ways: 1. the autopsies, performed by experienced veterinarians, could provide a unique opportunity for veterinary students to observe and learn the manual techniques necessary to reach and obtain specific organs and tissues; 2. any organ and tissue can be reached and samples can be obtained on demand, depending on the actual research interest; 3. also, on demand, the surgeon performing the autopsy can look for visible pathological changes, which could provide valuable information for the veterinarian, who had previously treated the dog, about the underlying mechanisms of the disease; 4. in addition, tissue specimens can be sent for more detailed pathological examinations, meanwhile they also allow scientists to search for molecular changes associated with the actual disease. With all these in mind, we have established the Canine Brain and Tissue Bank (CBTB), which accepts voluntary donations from owners of pet dogs, in cases when the euthanasia of the animal is advised by medical reasons. As the molecular quality of stored tissue samples is a major question, we first assessed this by measuring the gene expression stability of house keeping genes in brain and muscle collected under our protocol. We chose three endogenous reference genes that were previously validated in dog brain samples (*GAPDH, HPRT1* and *HMBS,* [23]). Our hypothesis was that the variance in the detected expression stability values of these genes, determined by BestKeeper and NormFinder, would be similar to the results shown by previous studies [23, 24], if the obtainment and purification procedures applied in the CBTB were efficient and met the quality standards. By investigating muscle samples in addition to brain tissue, we expected to find similar, or at least not greater, BestKeeper SD values, as reported previously for whole body tissues [24]. We chose temporal muscle as a second tissue type, because its solidity markedly differs from the brain, possibly affecting the efficacy of RNA purification from RNAlater fixed tissue pieces.

Materials And Methods

- Subjects in the current study

Brain and temporal muscle samples from five donated animals were included in the validation of the sampling protocol. All used samples were identified by the animal ID numbers and additional sample ID numbers (see Fig 2). The animals included in the validation study were the following: ID:170529–1: male Labrador retriever, 13 years old; ID:170713–1: male Labrador retriever, 14 years old; ID:170905–1 female

mongrel, 13 years old; ID:171102–1 female Beagle, 3 years old; ID:171102–2 female Beagle, 3 years old. Brain samples from one donated animal were used to assess the effect of RNAlater fixation on mRNA integrity: a male Labrador retriever, 13 years old (ID:180601–1).

- ○ ▪ Donation system

Samples of the Canine Brain and Tissue Bank (CBTB) originated from voluntary donations of owners whose dogs were euthanized due to medical reasons. The possibility of donations to the CBTB has been advertised through various social forums and media. Prior to the actual donation, both the owners and the veterinarians, who performed the euthanasia, had to fill in a “statement of donation” document, which contained a “statement of consent” to be filled by the veterinarian. Without that statement (which included statements of medical necessity and suitability) the donations were not accepted. Other criteria for accepting a donation included valid anti-rabies vaccination of the animal.

If the veterinarian / owner gave consent (by filling the appropriate field of the “statement of consent” document) for collecting and storing data about the animal, the medical records and any other known record (e.g. results of previous behaviour tests, questionnaires) were also obtained.

Euthanasia of the animals were performed at veterinary clinics and the cadavers were subsequently transported to the facility of the CBTB where the sampling was performed according to the predefined protocols. We set a 4 hours post-mortem delay as limit for sample fixation for molecular investigations, based on previous findings [25] and considering the expected maximal transportation times of donations from the clinics to the facility. The post-mortem interval between death of the animal and immersion of a tissue piece into RNAlater were recorded for every obtained sample (1 tissue piece = 1 sample).

- ○ ▪ Sampling and storage

For molecular research, brain samples were taken from the frontal cortex, cerebellum and brain stem, and muscle samples were collected from the temporal muscle, bilaterally (a total of eight samples / animal).

To obtain molecular grade tissue samples, 80 - 120 mg pieces from each site (e.g. a piece of cortex from the left side of the brain) were put into 1 ml RNAlater. After immersion in RNAlater, the samples were incubated overnight at 4°C before being frozen at –20°C. For long term storage, the RNAlater fixed tissue samples were transferred to –80°C following removal of the supernatant.

After obtaining the tissue pieces intended for molecular investigations, the two hemispheres of the brain were separated and stored under different conditions. The left hemisphere was put into 4% buffered formaldehyde and stored at 4°C. The right hemisphere was stored in an ULT freezer at –80°C degrees. In the current study, these samples (the formaldehyde fixed or frozen hemispheres) were not included. In the case of donations that exceeded the 4 hours time limit, samples were not included in the molecular research, and these brains (both hemispheres) were fixed in formaldehyde for histological purposes.

- ○ ▪ Experiment design

We performed two experiments to assess the quality of the obtained samples and the effect of the applied fixation / storage method.

In the first experiment, we assessed the stability of the three chosen reference genes in the three brain regions and muscle of five individuals using the samples collected and stored by our standard procedure (RNAlater fixation).

In the second experiment, we compared the Ct values of paired RNA samples derived from one animal: one half of the same tissue sample was put into RNAlater after removal from the body, while the other half was immediately put into TRIzol for RNA isolation. Therefore, we could assess whether RNAlater stabilization had an effect on the detected Ct values of the three housekeeping genes. Only brain samples (frontal cortex, cerebellum and brain stem, bilaterally) were used for this analysis.

- RNA isolation

Total cellular RNAs were isolated using TRIzol (Thermo Fisher Scientific) and following the manufacturer's protocol. For the isolation, smaller portions (30–50 mg) were cut from the RNAlater fixed tissue pieces and were rinsed in 1 ml sterile PBS before they being immersed in 500 ml TRIzol. Homogenization of the tissue pieces was done by an Ultra-Turrax homogenizer (Ika).

Quality of the isolates were checked by agarose gel electrophoresis, and the concentrations were measured by a NanoDrop device (Thermo Fisher Scientific). The isolates used for RT-qPCR all had a very similar S18/S28 ratio based on the gel electrophoresis results.

Isolated RNA samples were stored at –80°C for long term.

- cDNA synthesis and RT-qPCR

1000 ng of each total RNA was reverse-transcribed into cDNA using the Maxima RevertAid cDNA Synthesis Kit (Thermo Fisher Scientific) and following the manufacturer's protocol, with random hexamer primers. Prior to downstream applications, the cDNA samples were diluted tenfold by nuclease-free water and kept at –20°C. For long-term storage, the cDNA samples were placed at –80°C.

Quantitative Real-Time PCR reactions were performed on a StepOne Plus Instrument (Thermo Fisher Scientific) using commercial TaqMan assays and the TaqMan Gene Expression Master Mix (Thermo Fisher Scientific). The following TaqMan assays were used to target three genes, which were previously validated as reference genes for dog brain samples [23]: Cf04419463_gH for *GAPDH;* Cf02690456_g1 for *HPRT1;* Cf02694648_m1 for *HMBS.* Each reaction was performed in triplicate on a 96 well plate. Both non-template controls and negative controls were applied during the experiment. Negative controls contained non-transcribed RNA in the same dilution as in reaction mixes containing transcribed cDNA.

All reference genes were detected in all tissue samples tested. The efficiencies of the assays were determined by a five step dilution curve and were between 92% and 93% for all assays. Because of the

similar efficiency values, we assumed that the absolute expression levels of the genes were comparable in this setting.

Importantly, in some cases inter-assay comparisons showed more than 2 cycle threshold (Ct) differences between replicated measurements, while intra-assay relative values between different samples were more consistent, as shown by the example of *HPRT1 (*Fig. 1). The inter-assay average difference between same samples was found to be 3.03, while the inter-assay difference between relative Ct values (Ct of each sample compared to the average of each plate) was only 0.44. This was in accordance with the literature [26] regarding the higher consistency of relative Ct values.

As the capacity of plates used for our study allowed for a maximum of 96 reactions simultaneously, we assigned samples to provide the best grouping of parameters within the same plate in order to minimize the noise of inter-assay comparisons of absolute Ct values of the reference genes. For example, same tissue types were measured for all individuals on the same plate for each gene to minimize inter-assay noise of inter-individual differences.

Since the binding site of the assay primers can also modify results, because the 3' ends of mRNAs are more prone to degradation [27], we recorded the position of primer recognition sites on the mRNAs. In our study, the assays for *HPRT1* and *HMBS* recognized exons boundaries close to the 3' end, while the *GAPDH* assay was positioned in the middle of the reference mRNA sequence.

- ○ ▪ Software and statistical analysis

The absolute Ct means (derived from the three technical parallels run on a plate) belonging to different individuals were analysed in each tissue type to assess the stability of the reference genes. In the brain, comparisons were done in each investigated region (frontal cortex, cerebellum, brain stem) and for the whole brain altogether (regions grouped together).

We used MS Excel to determine standard deviations (SD), and we used the NormFinder [28] and BestKeeper [29] applications for detailed analyses of the stability of the reference genes.

We used the IBM SPSS Statistics software version 25 for statistical analysis. We used one-way ANOVA for pairwise comparisons of the absolute Cts belonging to each gene in all settings (3 brain region separately and together and muscle separately). As we analysed a total of five individuals, N was 5 for each gene in each region, except for the "whole brain" setting, where N was 15. Based on the number of genes, K was 3.

Results

- ○ ▪ Expression levels of the reference genes

First, we determined the relative expression of the three reference genes in the tissues. As the efficiency of the assays were similar for all genes, we could assume that the absolute Ct values were predictive of the

actual relative expression levels. Based on the means of absolute Ct values, *GAPDH* showed the highest and *HMBS* showed the lowest expression in all settings: in muscle, in each brain region, and when all brain regions were grouped together (Table 1). The difference between the genes' absolute Ct values were significant at $p < 0.001$ for all combinations (*GAPDH* vs. *HPRT1* vs. *HMBS)* in all settings, except for *HMBS* vs. *HPRT1* in brain stem, where we found no significant difference (Table 1). Our findings were in line with previous results, where *GAPDH* had the highest and *HMBS* had the lowest expression levels in the same tissues [23, 24, 30].

- ○ ▪ Expression stability of the reference genes

To answer the main question of our study, we first assessed the expression stability of these genes in brain samples, using the BestKeeper and NormFinder software to analyse the data. We found that *GAPDH* had the lowest BestKeeper SD (Table 2), when all brain regions were grouped together and in each brain region separately. In the same groupings, *HMBS* showed the second lowest SD and *HPRT1* had the highest SD by BestKeeper. Although the significance of differences between SDs could not be assessed in this setting, the order presented for the three genes by the BestKeeper SD values corresponded with the findings of Park et al. (2013). The correlation coefficient (r) determined by BestKeeper was the lowest for *HMBS* in our case, however, unfortunately, this parameter was not reported in the Park et al. (2013) paper.

In contrast to the BestKeeper analysis, NormFinder predicted *HMBS* to be the best reference gene (lowest "stability value"), when all brain regions were grouped together (Table 2). This finding was also in accordance with previous studies, which used NormFinder to analyse qPCR data from dog brain tissues [23, 24].

In muscle, we found *HPRT1* to have the smallest SD values (Table 2). On the other hand, *GAPDH,* which had the lowest BestKeeper SDs in the brain, was the most variable gene in this tissue. This finding was also in accordance with the results of Park et al. (2013), who also showed a marked difference in the BestKeeper SD of *GAPDH* when whole body tissues were grouped together and not only brain tissues were analysed. NormFinder predicted *HMBS* as the best reference gene in muscle (Table 2) and the stability values for all three genes in muscle were similar to stability values in brain tissue (no significant difference was shown between the two groups of values, see (Table 2).

When the means of the absolute Ct values measured in the two tissue types were compared, *GAPDH* showed higher expression in temporal muscle than in each brain region and in all brain regions together (differences were significant at $p < 0.01$) (Fig. 2). The differences between muscle and each brain region were also significant for *HMBS* ($p < 0.01$ for cerebellum and brain stem, $p < 0.05$ for frontal cortex), yet there was no significant difference in the case of *HPRT1* (Fig. 2). In concordance with this, the SD determined by BestKeeper was the lowest for *HPRT1* for all tissues grouped together, and NormFinder also predicted *HPRT1* as the best reference gene in this setting (Table 2).

- ○ ▪ Effect of sampling latency and RNAlater

We compared the post-mortem sampling latencies of each donation event with the Ct values. We found no correlation between sampling latency and mRNA abundance within the 4 hours post-mortem period (Fig. 3A-D).

In addition to assessing the variability of well-defined reference genes in our RNA isolates, we evaluated the effect of RNAlater treatment on mRNA quality in samples derived from one individual. As expected, our results indicated no significant differences between the Ct values of freshly isolated and RNAlater-treated sample pairs (Fig.3E).

Discussion

We have initiated a tissue bank to obtain and store tissue specimens from euthanized family pets using ethically acceptable protocols. To validate the molecular quality of tissue samples, we tested the expression of three endogenous reference genes in brain and temporal muscle tissues. Our results were consistent with the literature regarding the SD values calculated by BestKeeper in both tissue types [24]. The relative stability values, determined by NormFinder also corresponded with previous findings [23, 24], indicating that the efficiency of the method we used to collect tissues and purify RNA isolates was comparable with the procedures used by other research groups. It is important to note though, that in the current setting, we were not able to test the reliability of the results, by replicating the same experimental setting on another set of measurements. The SD values determined by BestKeeper always refer to a certain set of samples tested within the experiment, and even if both biological and technical parallels are used, the variance of SDs could be determined only by conducting independent experiments. In this regard, however, further gene expression studies based on the CBTB's tissue samples, will refine the current findings. For now, however, we can state, that we did not find any major divergences from the literature in the first line of our data. In addition, as we have showed that the stability of *GAPDH* was fundamentally different in brain and muscle tissue, our findings also strongly support previous recommendations [31] for RT-qPCR studies to apply more than one reference gene for reliable quantification, especially when several tissue types are included.

The incongruences we found between the predictions of BestKeeper and Normfinder were also acceptable according to previous reports. The utilization of different algorithms was previously reported by several authors to result in a somewhat variable ranking of reference genes. Nevertheless, the NormFinder ranking of the three genes in all brain tissues were consistent with both the Normfinder and GeNorm rankings presented by Stassen et al. (2015) and Normfinder ranking reported by Park et al. (2013), providing further confirmation for the reliable applicability of these three reference genes in canine gene expression studies.

To the best of our knowledge, only a few canine tissue banks have been established worldwide so far. Most of these serve primarily veterinary purposes, collecting tissues from donor animals for transplantational use [32, 33]. Existing canine tissue banks with primary scientific goals are represented by tumour banks, which store samples collected during standard veterinary practices [34]. However, in

these cases the range of obtainable tissues is limited by the tumour types and their healthy tissue counterparts, which are accessible for veterinarians during standard surgery practices. The CBTB could be a novel approach to provide scientists with organs and tissue types, which are normally not accessible from such a wide range of dogs (by breed, age, medical background etc.). If good quality and reliable documentation of all relevant factors are provided, these tissues could provide further insight into the epigenomes, transcriptomes and proteomes of dogs.

Especially gene expression studies could be highly relevant in many aspects, including disease and aging, to determine the underlying genetic mechanisms. Studies that investigate the expression of coding RNAs are fundamental to explore associations between expression patterns and complex traits [35–37] or biological (e.g. pathological or aging-related) changes [38–40]. Even minor alterations in the levels of the encoded proteins, which are involved in neuromodulation and neurotransmission, can have strong effect on behaviour [41–43] and may also play an important role in mental disorders in humans [44, 45]. Therefore, brain samples collected in a biobank could become indispensable to search for the gene expression patterns that correlate with canine behavioural variants. Also, as many canine behavioural abnormalities show high correspondences with their human counterparts, like aggressive tendencies [14], ADHD [46], obsessive-compulsive disorder [47, 48], and even autism spectrum disorder [49], findings about the genetic backgrounds of these phenotypes in dogs could also benefit humans. In this regard, it has already been demonstrated that genetic polymorphisms in neuroreceptor genes could lead to similar behavioural variation in the two species [43]. The genetic regulatory networks behind the wide range of breed-specific behaviours [50] could also be more easily unrevealed by comparative gene expression studies than by genome wide association studies. Most importantly, gene expression changes related to canine dementia could also be assessed this way and compared to human and rodent data already present in the literature [51–53]. As there is an increased interest towards dogs as natural models of human dementia, it is of high relevance to assess the similarities / differences in molecular level changes between the relevant species, which has already been done for mice and humans [54]. In addition, dogs can suffer from several other disorders, which affect the brain and still remain barely understood, limiting the range of potential treatments. In humans, recent advances have led to the increasing utilization of gene expression data to help understand the genetic background of complex diseases [55, 56]. The same approach could be applied for canine disorders too, provided that researchers can get access to biological materials that allow reliable gene expression profiling. Importantly, as several genetic disorders occur only in certain breeds, canine tissue banks should become able to collect and store samples from a large number of dogs, which represent a wide range of breeds. For example, epilepsy is highly prevalent in many breeds [57–59], indicating a strong genetic predisposition in these populations. However, the causative variants and molecular changes that lead to epilepsy are barely understood in dogs. Comparative gene expression analyses of brain tissues obtained from affected and non-affected dogs in a certain breed could facilitate the identification of genetic risk factors.

Conclusions

On the long run, a successfully established canine tissue bank may also facilitate the establishment of similar initiatives across the world, leading to possible collaborations. This is common practice in human brain banks [18]. For this purpose, however, quality standards have to be set in the beginning and should be continuously improved to reach a state where the protocol can be integrated by other research groups. Evaluation of re mRNA quality of abundant reference genes was a first step to ensure that samples stored in the Canine Brain and Tissue Bank will suffice future molecular research demands both in translational and veterinary fields.

Declarations

- Ethics approval

Research and sample collection done by the Canine Brain and Tissue Bank does not involve live animals. At the time of developing the donation system and sampling protocol for the CBTB, the National Food Chain Safety Office (NFCSO) was asked to provide approval. They stated, no specific approval was necessary, as no live animals would be involved in the process. Only approval to transport animal carcasses was necessary and so it was obtained.

- ◦ ▪ Consent for publication

Not applicable.

- ◦ ▪ Data availability

All data generated or analysed during this study are included in this published article (and its supplementary information files).

- ◦ ▪ Competing interests

The authors declare that they have no competing interests.

- ◦ ▪ Funding

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- ◦ ▪ Authors’ contributions

SS participated in sample acquisition, sample processing, molecular procedures (RNA purification and RT-qPCR), analysed and interpreted the data, and wrote the manuscript. KC performed the autopsies for the sampling and participated in writing the manuscript, with special regard to the veterinary aspects. KT participated in sample processing and molecular procedures and contributed to writing the manuscript.

EK contributed to writing the manuscript and analysing the data. All authors read and approved the final manuscript.

- ○ ▪ Acknowledgements

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Tables

Table 1.

	descriptive stats						p values				
Region or tissue	*GAPDH* mean	*GAPDH* SD	*HPRT1* mean	*HPRT1* SD	*HMBS* mean	*HMBS* SD	*GAPDH* vs *HPRT1*	*GAPDH* vs *HMBS*	*HPRT1* vs *HMBS*	ANOVA critical F	F value
Frontal cortex	20.8313	0.49530	25.7709	0.64006	27.7143	0.52261	< 0.001	< 0.001	< 0.001	12.97	203.512
Cerebellum	19.9549	0.19141	26.1186	0.38806	27.3677	0.37468	< 0.001	< 0.001	< 0.001	12.97	721.109
Brain stem	20.1067	0.42351	26.5321	0.91335	27.1921	0.76749	< 0.001	< 0.001	0.179	12.97	143.395
Brain together	20.2977	0.53711	26.1405	0.70861	27.4247	0.58043	< 0.001	< 0.001	< 0.001	8.25	575.930
Temporal muscle	18.7292	1.04104	25.4488	0.55177	28.7740	0.67636	< 0.001	< 0.001	< 0.001	12.97	212.804

Table 1. Descriptive statistics of gene expression

Basic descriptive statistics were determined by MS Office and ANOVA was performed by IBM SPSS version 25.

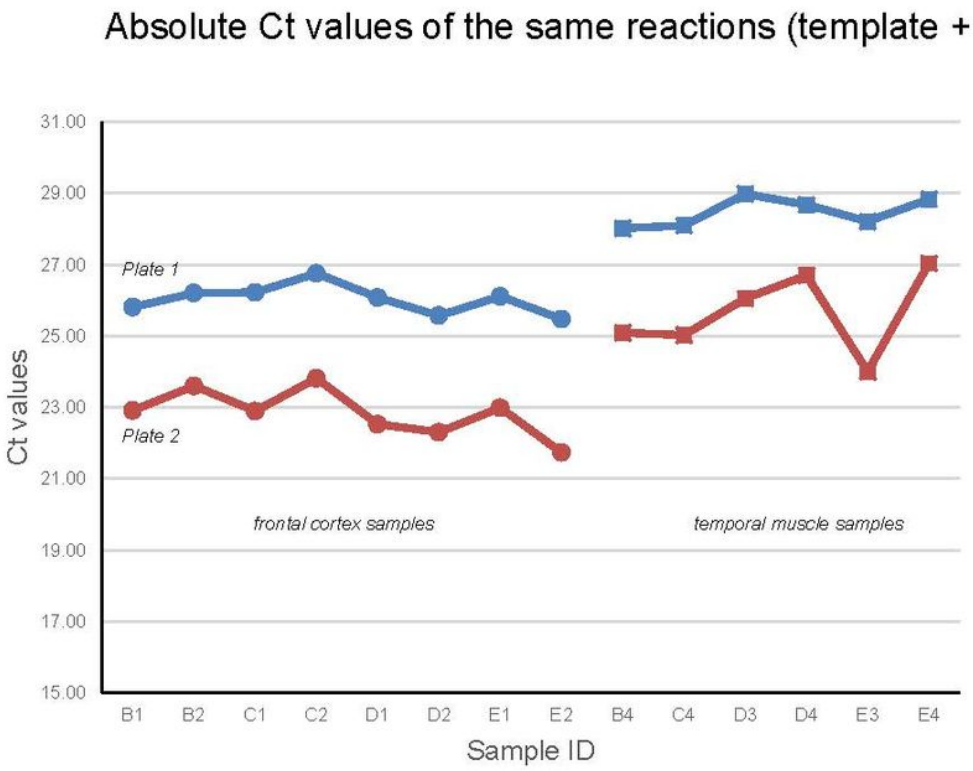
Table 2.

Sample type	Lowest SD of Cts (SD in brackets)	Best gene by NormFinder (stability values in brackets)	Second best gene by NormFinder (stability values in brackets	Third best gene by NormFinder (stability values in brackets	Best pair of genes by NormFinder	BestKeeper lowest SD (SD in brackets)	BestKeeper highest corr. coeff. (value in brackets)
Frontal Cortex	*GAPDH* (0.5)	*GAPDH* (0.054)	*HMBS* (0.130)	*HPRT1* (0.259)	n/a	*GAPDH* (0.35)	*GAPDH* (0.862)
Cerebellum	*GAPDH* (0.19)	*HPRT1* (0.026)	*HMBS* (0.055)	*GAPDH* (0.074)	n/a	*GAPDH* (0.15)	*HPRT1* (0.979)
Brain stem	*GAPDH* (0.42)	*HMBS* (0.041)	*HPRT1* (0.094)	*GAPDH* (0.119)	n/a	*GAPDH* (0.32)	*GAPDH* (0.994)
Temporal muscle	*HPRT1* (0.55)	*HMBS* (0.076)	*GAPDH* (0.183)	*HPRT1* (0.267)	n/a	*HPRT1* (0.4)	*GADPH* (0.964)
Brain together	*GAPDH* (0.54)	*HMBS* (0.087)	*GAPDH* (0.121)	*HPRT1* (0.147)	GAPDH and HPRT1	*GAPDH* (0.46)	*HMBS* (0.872)
All tissues	*HPRT1* (0.72)	*HPRT1* (0.194)	*GAPDH* (0.201)	*HMBS* (0.230)	GAPDH and HMBS	*HPRT1* (0.54)	*GAPDH* (0.828)

Table 2.

The main parameters associated with the reference genes' stability by various approaches are shown in the table. Values of the parameters are shown in brackets.

Figures



Sample ID	tissue	Animal ID
B1	frontal cortex	170713-1
B2	frontal cortex	170713-1
B4	temporal muscle	170713-1
C1	frontal cortex	170905-1
C2	frontal cortex	170905-1
C4	temporal muscle	170905-1
D1	frontal cortex	171102-1
D2	frontal cortex	171102-1
D3	temporal muscle	171102-1
D4	temporal muscle	171102-1
E1	frontal cortex	171102-2
E2	frontal cortex	171102-2
E3	temporal muscle	171102-2
E4	temporal muscle	171102-2

Figure 1

Inter-assay differences in Ct values The figure depicts HPRT1 as tested on separate plates in frontal cortex and temporal muscle samples. The same reactions (template sample + target) were loaded in three technical parallels on both plates. The samples included in this measurement are listed in the table on the right. Relative Ct values were defined as the difference between each sample's Ct mean (three technical parallels together) and the average Ct of all samples from the same tissue.

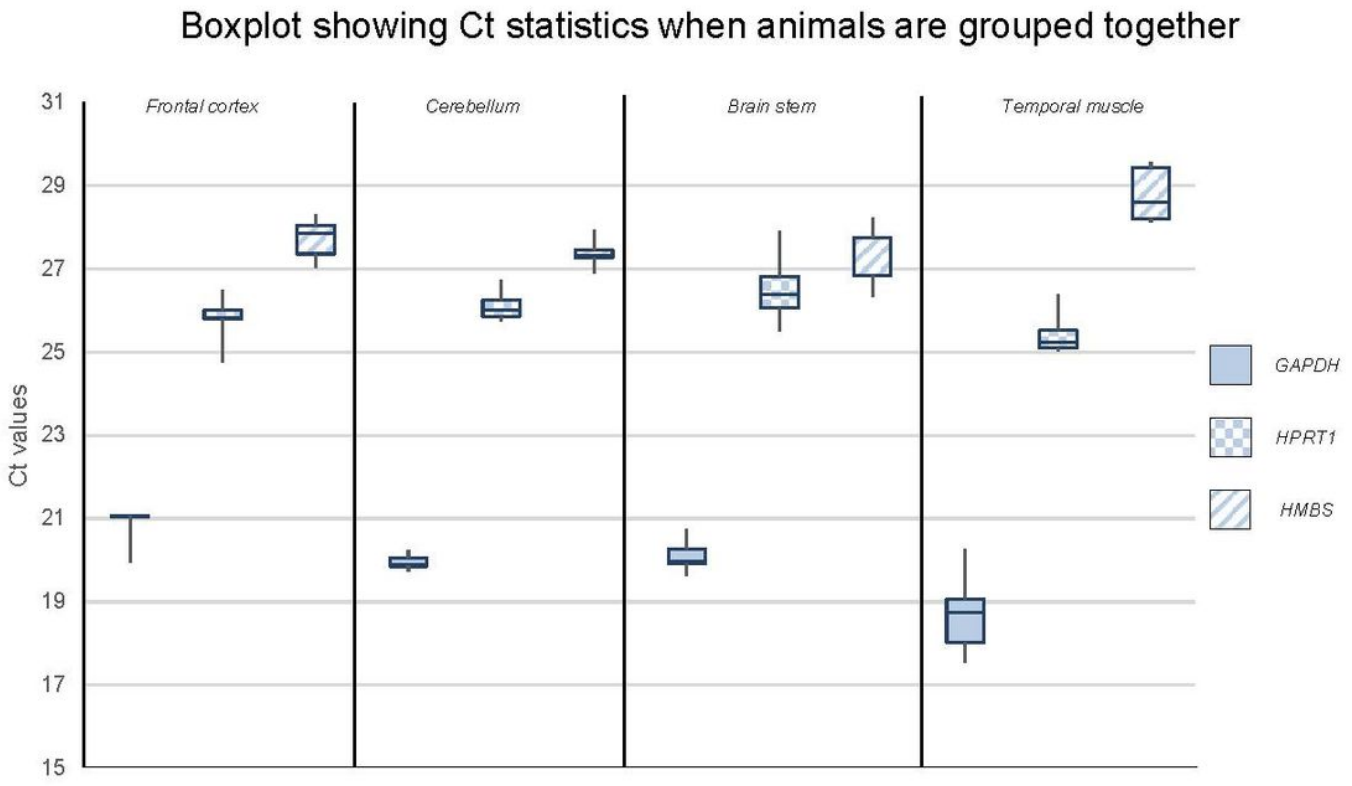


Figure 2

Boxplot showing Ct statistics for each gene in different tissues The figure illustrates variance of Ct values in all investigated tissues for all genes. In this setting, inter-assay variance may have affected the detected difference between tissues as samples belonging to the same tissue were grouped together in separate plates and not all samples were measured on more than one plate. However, the differences between animals – which was the main question of the study – could be measured on an intra-assay comparison because of the grouping. Significance levels of pairwise differences are not indicated on the figure to maintain clarity. All significant differences are listed below: • GAPDH frontal cortex vs. cerebellum p = 0.040 • GAPDH temporal muscle vs. frontal cortex p = 0.001 • GAPDH temporal muscle vs. cerebellum p = 0.007 • GAPDH temporal muscle vs. brain stem p = 0.003 • HPRT1 temporal muscle vs. brain stem p = 0.018 • HMBS temporal muscle vs. frontal cortex p = 0.014 • HMBS temporal muscle vs. cerebellum p = 0.002 • HMBS temporal muscle vs. brain stem p = 0.001

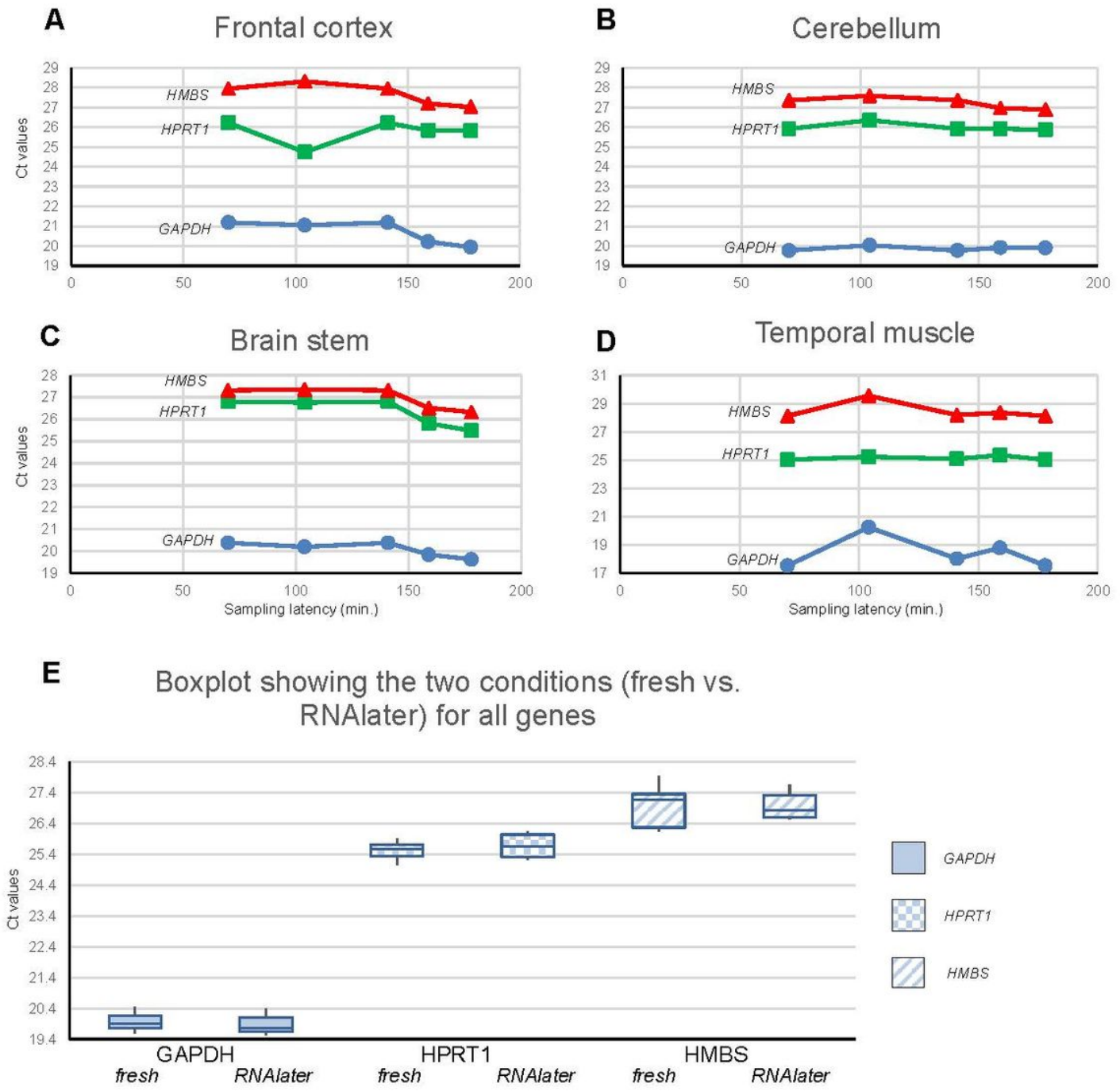


Figure 3

Ct values plotted against post-mortem delay of sampling The figure depicts the average Ct values of all target genes in all tissues plotted against the post-mortem sampling latencies of each sample. Post mortem sampling latencies were measured as the time elapsed between death of the animals and RNAlater fixation of the removed tissue pieces. A: frontal cortex; B: cerebellum; C: brain stem; D: temporal muscle. Blue circles = GAPDH; Green squares = HPRT1; Red triangles = HMBS