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Lateralization of housekeeping genes in the brain of one-day old chicks

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ABSTRACT

Real-time quantitative PCR is an exceptionally sensitive method that can detect even very small differences in gene expression and, as such, it is essential to use suitable reference genes. Domestic chickens are used in a wide range of studies including neurobiology, behavior, ecology and disease transmission. In recent avian gene expression experiments, 18S (18S ribosomal RNA), beta actin (ACTB) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) have frequently been used; however, there is not enough evidence that these reference genes are suitable for all types of experiments. There is considerable evidence for lateralization in numerous learning tasks and for differences in the functional contribution of the two brain hemispheres. Therefore, the purpose of this study was to identify a set of reference genes for chick brain region called an intermediate medial mesopallium (IMM), which is connected with memory formation in the chick brain, whilst also taking into consideration the differences between the left and right hemispheres. This study evaluated the expression stability of eleven candidate housekeeping genes in the IMM region of the 1-day old chick brain. In our experimental system, the most reliable results were given by the NormFinder algorithm. The results show for the first time that ACTB, commonly used as an avian reference gene, is not suitable for investigation of gene expression in the chick brain and that brain lateralization exact selection of different reference gens for each hemisphere. For memory process investigations using tasks in one-day old chicks the most effective reference genes for the left hemisphere were HMBS and SDHA, and for the right hemisphere the most effective was RPL19.

1. Introduction

Real-time quantitative PCR is an exceptionally sensitive method that allows for the detection of even very small differences in gene expression. For this reason, it is also very sensitive to various kinds of errors and inaccuracies. In experimental systems the most common mistake is to use only one reference gene, usually ACTB (beta actin) or GAPDH (glyceraldehyde 3-phosphate dehydrogenase) without prior validation (Chapman and Waldenström, 2015). For model organisms such as humans, rats or mice there are commercially available panels of reference genes; however, the information concerning reference genes in non-mammalian vertebrates is limited. For instance, the data available for the domestic chicken (*Gallus gallus*) are very modest (Gibbs, 2008; Moorman and Nicol, 2015). Domestic chickens are used in a wide range of studies, such as in neurobiology, behavior, ecology, and disease transmission. In recent avian gene expression experiments 18S (18S ribosomal RNA), ACTB and GAPDH have been used frequently (Olias et al., 2014), but there is not enough evidence to show that these reference genes are suitable for all types of experiments.

There is a strong evidence that implicates the intermediate medial mesopallium (IMM; formerly known as the intermediate medial hyperstriatum ventrale - IMHV) in visual imprinting and avoidance learning which in chicks is a process common to the young animals. Therefore experimental tasks are usually performed on one-day old chicks of both sexes because at this age gender does not influence the task (Nakamori et al., 2013; Rose, 2000). The biochemical and morphological changes are more prevalent in the left IMM than in the right, and the observed changes show time-dependent shifts in the location (Rose, 2000). There is considerable evidence for lateralization in numerous learning tasks and for differences in the functional contribution of the two brain hemispheres (Moorman and Nicol, 2015). Recent studies have shown

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that using single or inappropriate reference genes for normalization may dramatically alter the results of mRNA copy number quantification and that commonly used reference genes, including those used for chicks, do not accomplish the task (Olias et al., 2014). Therefore, it is clear that a new set of reference genes need to be established for experiments where lateralization plays a crucial role in the processes being investigated.

The purpose of this study was to identify a set of reference genes for a memory connected IMM brain region in the chick brain, taking into consideration the basic differences in the left and right hemispheres.

2. Results

2.1. Expression profiling of candidate reference genes

The ranges of the Cq values, expressed as the mean average of the replicates, for each candidate reference gene in both studied groups (IMM from the left and from the right hemisphere) were represented in the form of boxplots (Fig. 1A and B) The results show that ACTB, GAPDH and YWHAZ were the most expressed genes in both the left and the right hemispheres. GUSB, HMBS and HPRT1 were the least expressed genes, with the highest mean Cq-values. Each candidate reference gene did show some expression variation, however, some of them exhibited an unacceptably high variation making them unsuitable for use as a reference gene. Generally, the left and right hemispheres show a similar pattern of eleven candidate reference genes expression, but a detailed analysis using the GeneEx 6.1 software shows differences between them.

2.2. geNorm analysis

The geNorm module of the GeneEx 6.1 software calculates a gene stability measure, M, which is the average pairwise variation of the expression level of one particular reference gene compared to all the other genes tested. Lowering of the M value denotes an increase in gene stability, accordingly, the most stable reference gene is the one with the lowest M value. In our sample panel, geNorm analysis revealed that in the left hemisphere PGK1 and HPRT1 were the most stable reference genes with M = 0,139667 (Fig. 1C). Unexpectedly, the commonly used reference gene ACTB was found to be the least stable gene, with M = 0,664699. In the right hemisphere the most stable reference genes were HPRT1 and HMBS (M = 0,1610445) and, again, ACTB was found to be the least stable gene (Fig. 1D). The average M values for the eleven tested reference genes in both the left and right hemispheres are presented in Table 1. HPRT1 was found to be the best reference gene for both the left and right hemisphere of the one-day old chick brain.

2.3. NormFinder analysis

The same dataset was evaluated with the NormFinder module. This algorithm ranks the set of candidate genes according to their expression stability value in a given sample set and a given experimental design. In the left hemisphere HMBS and SDHA were defined as the most stable genes (Fig. 1E). In the right hemisphere the most stable reference gene was RPL19 (Fig. 1F).

The NormFinder algorithm available in the GenEx 6.1 software can also determine the optimal number of control genes to be used in the normalization process by calculating the Accumulated Standard Deviation. (Acc.SD). The Acc. SDs of the eleven reference genes for the left hemisphere are shown in Fig. 1G and for the right hemisphere in Fig. 1H. The optimal number of reference genes is indicated by the lowest value for the Acc. SD. The lowest value for the left hemisphere was observed when two reference genes, HMBS and SDHA, were used. Overall, then, the use of just HMBS or SDHA as reference genes for IMM from the left hemisphere of the chick brain appears to be sufficient for good normalization. The Acc. SD calculated by the NormFinder for the right hemisphere indicated that the use of only one reference gene – RPL19 - was optimal.

3. Discussion

The most common method of data normalization in qPCR experiments is the use of stably expressed reference genes. The ideal reference gene should be unaffected by the experimental treatment and developmental stage. The literature review by Chapman (Chapman and Waldenström, 2015) demonstrates that many researchers continue to use a single, invalidated reference gene to normalize their data. Such a procedure is against the rules developed for researchers performing gene expression analyses, and can lead to misinterpretation of the data and false conclusions. At least seven programs to calculate the most stable reference genes are now available (geNorm, NormFinder, BestKeeper, RefFinder and the R-based packages: NormqPCR, SLqPCR and NormFinder for R) (Bustin et al., 2009). There is certainly no need to use all above-mentioned programs at once during routine gene expression analysis. The pairwise correlation of the geNorm algorithm is known to be a strong algorithm for small sample sizes, but tends to select genes that are mutually correlated. The NormFinder can differentiate intragroup variation from intergroup variation and is therefore a suitable tool for identifying candidate genes when different sample groups are to be assessed, but it requires larger sample sizes compared to geNorm (De Spiegelaere et al., 2015). Vertebrate embryos exhibit a strikingly conserved left-right (LR) asymmetry of the internal organs. This asymmetry is maintained in adults and extends to the brain and nervous system (Levin, 2004). In the brain, the left and right hemispheres are anatomically asymmetric and have distinctive functions, although the molecular basis for this asymmetry has not yet been characterized. The human brain exhibits asymmetry in both macroscopic and microscopic level. Many studies have revealed the anatomical left-right asymmetry in the size of regions involved in language and auditory processing, such as planum temporale, sylvian fissure, and Heschl's gyrus (Dorsaint-Pierre et al., 2006; Rubens et al., 1976; Schneider et al., 2005).

Some decades ago, it was assumed that brain asymmetry was unique to the human brain only. The lack of symmetry in human barins was connected with plasticity processes, which are critical for human cognitive evolution (Gómez-Robles et al., 2013). Now it is well known that not only primates (humans and chimpanzees) (Gómez-Robles et al., 2016) but most other vertebrates, for example cats (Webster and Webster, 1975) and even eight species of Australian parrots (Magat and Brown, 2009) have strong left–right asymmetries in their brains. A strong odor dependence of the lateralization of short-term memory recall of odors has been reported in honeybees (Rigosi et al., 2011).

Recent studies, performed using modern computer technics, confirmed the phenomenon of brain anatomical asymmetry (Wachinger et al., 2015). Asymmetry affects not only healthy people, but also manifests strongly in a number of pathological conditions such as dementia (Wachinger et al., 2016) or Autism Spectrum Disorders (Carper et al., 2016). Brain asymmetry and lateralization has also been observed at the biochemical level. Proteomic analysis has shown differential protein expression in the hippocampi (left vs. right) of young adult male rats. The expression of Dynamin-1, DRP2, synapsin-1 was higher in the right hippocampus than in the left (Samara et al., 2011). Differential gene