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HOUSEKEEPING GENES REVISITED: DIFFERENT EXPRESSIONS DEPENDING ON GENDER, BRAIN AREA AND STRESSOR

N. M. DERKS,* M. MÜLLER, B. GASZNER,[1] D. T. W. M. TILBURG-OUWENS, E. W. ROUBOS AND L. T. KOZICZ

Department of Cellular Animal Physiology, Radboud University Nijmegen, P.O. Box 9010, 6500 GL Nijmegen, The Netherlands

Abstract—Housekeeping gene (HKG) mRNAs are used to normalize expression data of genes of interest in quantitative reverse transcriptase polymerase chain reaction studies. Such normalization assumes constant HKG gene expression under all circumstances. Although sporadic evidence suggests that HKG expression may not always fulfill this requirement and, therefore, such normalization may lead readily to erroneous results, this fact is generally not sufficiently appreciated by investigators. Here, we have systematically analyzed the expression of three common HKGs, glyceraldehyde-3-phosphate dehydrogenase, ribosomal subunit 18S and β-actin, in two different stress paradigms, in various brain areas, in male and in female rats. HKG expressions differed considerably with respect to brain area, type of stressor and gender, in an HKG-specific manner. Therefore, we conclude that before final experimentation, pilot expression studies are necessary to select an HKG which expression is unaffected by the experimental factor(s), allowing reliable interpretation of expression data of genes of interest.

Key words: q-RT-PCR, housekeeping genes, brain area, gender, stressor, adaptation.

Normalization of quantitative reverse transcriptase polymerase chain reaction (q-RT-PCR) data of a gene of interest to that of a 'housekeeping gene' (HKG) is commonly considered a crucial step in accurately determining gene expression levels in experimental designs (Suzuki et al., 2000; Tanic et al., 2007). The 'ideal' HKG will be expressed in all cells of an organism and this expression will be constant under all circumstances and among all tissues (Thellin et al., 1999). However, recent studies suggest that expression of HKGs can vary depending on the experimental design. For example, during brain development, the degree of expression of the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene changes whereas that of the ribosomal subunit 18S remains constant (Al-Bader and Al-Sarraf, 2005). In contrast, when adult rats were subjected to dietary restrictions, GAPDH was found to be a stable HKG for accurate normalization, while under this condition 18S expression appeared to be regulated (Tanic et al., 2007). In addition, stress induces 28S rRNA expression in the amygdala (Maeda et al., 2005) and glucocorticoid treatment affects 18S rRNA expression in the cerebral cortex and hippocampus (Tanic et al., 2007). In all these cases normalization may lead readily to erroneous results (Thellin et al., 1999; Dheda et al., 2004), a notion, however, that is generally not sufficiently appreciated by investigators.

In this study, we analyzed the (lack of) constancy in the expression of the HKGs GAPDH, 18S and β-actin, in two different stress paradigms (acute and chronic stress), in various brain areas, viz. the hypothalamic paraventricular nucleus (PVN), hippocampus, central nucleus of the amygdala (CeA), oval nucleus of the bed nucleus of the stria terminalis (BNSTov), prefrontal cortex (PFC), motor cortex (MoC), Edinger-Westphal nucleus (EW) and cerebellum (Ce), in male and female (in random phases of the estrous cycle) rats.

EXPERIMENTAL PROCEDURES

Animals

Wistar-R Amsterdam rats (225–250 g, light/dark 12/12 h; lights on 07:00 h) were housed under standard vivarium conditions in three groups, acutely stressed, chronically stressed and control rats, each group consisting of six males and six females. For acute stress, rats were placed in a plastic restrainer (length 200 mm, diameter 45 mm, several ventilation holes) for 1 h. For chronic stress, rats were exposed to 'chronic variable stress' for 14 days (after Marin et al., 2007; see Table 1). Control animals were handled as challenged rats, but not stressed. Studies were conducted in accordance with the Declaration of Helsinki and the animal use guidelines from the Medical Faculty Committee for Animal Resources of Pécs University, Pécs, Hungary. All efforts were taken to minimize the number of animals and the extent of their suffering.

RNA extraction and cDNA synthesis

Frozen brains were cut using a coronal brain matrix (Ted Pella, Redding, CA, USA; no.: 15007) between the Ce and both hemispheres. Then 1 mm-thick coronal sections were made with a razor blade. The brain slices were placed on a chilled mat, and the areas were punched out with a Harris Unicore Hole 1.0 mm puncher (Ted Pella). Punched samples were collected in 500 μl ice-cold Trizol (Life Technologies, Paisley, UK) and homogenized by sonification. After chloroform extraction and isopropyl alcohol precipitation, RNA was dissolved in 30 μl RNAse-free DEPC. Total RNA was measured with an Eppendorf Biophotometer (Vaudaux-Eppendorf AG, Basel, Switzerland). First strand cDNA synthesis was performed using 1 μg RNA dissolved in 11 μl RNAse-free DEPC containing 0.25 mU pd(N)6 (random primers;

[1] Present address: B. Gaszner, Department of Anatomy, Medical Faculty, Pécs University, H-7643 Pécs, Szigeti út 12, Hungary.

*Corresponding author. Tel: +3124-3652705; fax: +3124-2652714.
E-mail address: nm.derks@science.ru.nl (N. M. Derks).

Abbreviations: BNSTov, oval nucleus of the bed nucleus of the stria terminalis; Ce, cerebellum; CeA, central nucleus of the amygdala; Ct, cycle threshold; EW, Edinger-Westphal nucleus; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HKG, housekeeping gene; MoC, motor cortex; PFC, prefrontal cortex; PVN, hypothalamic paraventricular nucleus; q-RT-PCR, quantitative reverse transcriptase polymerase chain reaction.

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Table 1. Variable stress protocol

Day	Stress type
1	Swim stress, 2 min (4 °C); humid sawdust, 3 h
2	Food/water deprivation, permanent
3	Lights on, overnight; humid sawdust, permanent
4	Lights off, 180 min; swim stress, 2 min (4 °C)
5	Food/water deprivation, overnight; isolation, overnight
6	Cold isolation (4 °C), 15 min; lights off, 120 min
7	Swim stress, 4 min (12 °C); food/water deprivation, overnight
8	Inverted light/dark cycle; humid sawdust, overnight
9	Constant light, overnight; food/water deprivation, overnight
10	Lights off, 180 min; humid sawdust, permanent
11	Isolation, overnight; food/water deprivation, overnight
12	Restraint stress, 60 min; lights on, overnight
13	Inverted light/dark cycle; food/water deprivation, overnight
14	Humid sawdust, 3 h; restraint stress, 60 min

Roche, Almere, The Netherlands) at 70 °C for 10 min, followed by double-strand synthesis in 1× strand buffer (Life Technologies) with 10 mM DTT, 20 U Rnasin (Promega, Madison, WI, USA), 0.5 mM dNTPs (Roche) and 100 U reverse transcriptase (Superscript II; Life Technologies), at 37 °C for 75 min and at 95 °C for 10 min. RNA concentration was measured with the NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA). The A260/280 ratio was in all samples between 1.8 and 2.0, indicating high purity. To check for RNA integrity, 500 ng of RNA was loaded onto a 1% agarose gel with GelRed Nucleic Acid Gel Stain (Biotium, Hayward, CA, USA), subjected to electrophoresis, and visualized by UV transillumination. There appeared to be no RNA degradation as indicated by intact 28S and 18S ribosomal RNA bands in all samples.

q-RT-PCR

Quantitative RT-PCR was performed in a total volume of 25 μl buffer solution containing 5 μl of template cDNA, 12.5 μl SYBR Green Master mix (Applied Biosystems, Foster City, CA, USA), 1.5 μl DEPC-treated MQ and 15 μM of each primer. Primers for the HKG GAPDH, β-actin and 18S were designed using Vector PrimerExpress software (Applied Biosystems) based on the rat cDNA sequence for GAPDH (acc. number M32599), β-actin (acc. number NM 031144) and 18S (acc. number M11188). Primer pairs were for GAPDH: 5′-TGCCATCACTGCCACTCAGA-3′ and 5′-GTCAGATCCACAACGGATACATTG-3′, for β-actin: 5′-GTAACCCGTTGAACCCCATT-3′ and 5′-CCATCCAATCGGTAGTAGCG-3′ and for 18S: 5′-TTGCTGATCCACATCTGCTGG-3′ and 5′-ATTGCCGACAGGATGCAGAA-3′. The optimum temperature cycling protocol was determined to be 95 °C for 10 min followed by 35 reaction cycles at 95 °C for 15 s and at 60 °C for 1 min, using a 7500 GeneAmp PCR system (Applied Biosystems). For each reaction, the cycle threshold (Ct) was determined, i.e. the number of cycles needed to detect fluorescence above the arbitrary threshold (0.8). At this threshold Ct-values are within the exponential phase of the amplification. Standard curves were included in duplo with cDNA-concentrations ranging from 6.25 ng to 100 ng cDNA content. Using these curves, where every Ct-value corresponds to a certain amount of cDNA, the quantity of cDNA was calculated for each sample with Applied Bioscience 7500 System Software. Efficiencies of the primer pairs were for GAPDH: 1.97 (r^2=0.991), for β-actin: 1.94 (r^2=0.996) and for 18S: 2.04 (r^2=0.999).

Statistics

Differences between group mean values were tested by multiple analysis of variance and Student's *t*-test, after testing for normality (Shapiro and Wilk, 1965) and homogeneity of variance (Bartlett's chi-square test; Snedecor and Cochran, 1989) and followed by Fisher's LSD post hoc test using Statistica (StatSoft, Tulsa, OK, USA).

RESULTS

Our data revealed that the reaction of an HKG mRNA strongly depended on the type of stressor, the brain area and the gender. Moreover, the relations of the three HKG mRNAs to these three factors were quite different from each other. This is clear from the following results.

As to the *type of stressor:* in the PVN, acute stress increased β-actin in both males (+43%; $F_{1,9}$=0.83; P<0.05) and females (+28%; $F_{1,10}$=0.88; P<0.05), whereas chronic stress increased this HKG only in females (+29%; $F_{1,10}$=0.95; P<0.05). Furthermore, in the female BNSTov, acute stress increased the expression of β-actin (+42%; $F_{1,10}$=0.73; P<0.05) and 18S (+41%; $F_{1,10}$=0.33; P<0.05) but chronic stress had no effect on these HKGs. In the MoC, chronic stress increased GAPDH in females (+30%; $F_{1,10}$=0.79; P<0.05) and decreased 18S in both males (−22%; $F_{1,9}$=0.23; P<0.05) and females (−23%; $F_{1,10}$=0.36; P<0.05), but acute stress had no effect on these HKG in this area, neither in males nor in females. In the male hippocampus, acute stress increased β-actin expression (+50%; $F_{1,9}$=0.73; P<0.05) but chronic stress did not show any effect on this HKG. The different actions of the two stressors also appeared in the male PFC, where GAPDH expression was +48% higher ($F_{1,9}$=0.09; P<0.05) after chronic than after acute stress.

As to *gender:* in the female PVN upon chronic stress, β-actin and 18S expressions were +40% ($F_{1,10}$=0.38; P<0.05) and +27% ($F_{1,10}$=0.13; P<0.05), respectively, higher than in males. In the BNSTov, GAPDH expression in control males was higher than in control females (+28%; $F_{1,9}$=0.60; P<0.05). In the PFC, GAPDH expression was lower in control (−38%; $F_{1,9}$=0.35; P<0.05) and chronically stressed (−32%; $F_{1,10}$=0.07; P<0.05) females than in the corresponding males. Moreover, after acute stress, GAPDH expression in the EW was much higher in males (+56%; $F_{5,30}$=0.18; P<0.05) than in females. In the hippocampus, 18S expression was lower in control males than in control females (−20%; $F_{1,9}$=0.29; P<0.05).

As to *brain area:* in the hippocampus, acute stress stimulated only β-actin expression (males: +33%; F=0.73; P<0.05) while in the PFC this stressor increased only GAPDH (females: +52%; F=0.65; P<0.05). Furthermore, the BNSTov was only affected by acute stress whereas the MoC was only affected by chronic stress (see also above). More generally, whereas most brain centers revealed (varying degrees of) stressor- and/or gender-specific HKG mRNA expression dependences, the CeA and Ce did not show any dependence at all.

These and more differences between the three HKG mRNAs in their stressor-, brain area- and gender-specific expressions are summarized in Fig. 1.

DISCUSSION

The aim of the present study is to draw attention to the fact that neuronal proteins generally considered (and utilized)