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Sensitivity of housekeeping genes in the suprachiasmatic nucleus of the mouse brain to diet and the daily light–dark cycle

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ABSTRACT

The endogenous timing system within the suprachiasmatic nuclei (SCN) of the hypothalamus drives the cyclic expression of the clock molecules across the 24 h day–night cycle controlling downstream molecular pathways and physiological processes. The developing fetal clock system is sensitive to the environment and physiology of the pregnant mother and as such disruption of this system could lead to altered physiology in the offspring. Characterizing the gene profiles of the endogenous molecular clock system by quantitative reverse transcription polymerase chain reaction is dependent on normalization by appropriate housekeeping genes (HKGs). However, many HKGs commonly used as internal controls, although stably expressed under control conditions, can vary significantly in their expression under certain experimental conditions. Here we analyzed the expression of 10 classic HKG across the 24 h light–dark cycle in the SCN of mouse offspring exposed to normal chow or a high fat diet during early development and in postnatal life. We found that the HKGs glyceraldehyde-3-phosphate dehydrogenase, beta actin and adenosine triphosphate synthase subunit to be the most stably expressed genes in the SCN regardless of diet or time within the 24 h light–dark cycle, and are therefore suitable to be used as internal controls. However SCN samples collected during the light and dark periods did show differences in expression and as such the timing of collection should be considered when carrying out gene expression studies.

Abbreviations: HKG, housekeeping genes; SCN, suprachiasmatic nucleus; *Atp5b*, adenosine triphosphate synthase subunit; *B-Act*, beta actin; *B2M*, beta-2-microglobulin; *Cyc1*, cytochrome c-1; *Can*, Calnexin; Eif4a2, eukaryotic translation initiation factor 4A isoform 2; *Gapdh*, glyceraldehyde 3-phostphate dehydrogenase; *Sdha*, succinate dehydrogenase complex subunit A; *Ubc*, ubiquitin C; *Ywhaz*, tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein

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1. Introduction

Most mammals have an endogenous timing system in the suprachiasmatic nuclei (SCN) of the hypothalamic region of the brain. It consists of an intracellular feedback loop coordinating the expression of molecular transcripts (termed as 'clock' genes and clock-controlled genes) and their constitutive protein to oscillate across the 24 h day–night cycle (Buhr and Takahashi, 2013; Cagampang and Bruce, 2012). These 24 h oscillations bring about rhythmic changes in downstream molecular pathways and physiological processes, as well as overt rhythmic changes in behavior such as the sleep–wake cycle, locomotor activity and feeding. Environmental cues or Zeitgebers, such as the daily light–dark cycle, temperature and feeding cycles, are able to entrain this 'clock' system to maintain its integrity and temporal coordination (Panda and Hogenesch, 2004; Roenneberg et al., 2007). Studies are now emerging that similar clock systems are also be found in non-SCN neurons and in peripheral tissues, including the heart (Peirson et al., 2006; Young et al., 2001)and the liver (Davidson et al., 2004; Mohawk et al., 2012; Peirson et al., 2006). Nevertheless, signals from the 'central' clock in the SCN are required to maintain daily rhythms in these tissues.

Disrupting the integrity and temporal coordination of the clock system, in the SCN and peripheral tissues, can lead to hormonal imbalances, sleep disorders, cardiometabolic diseases, and susceptibility to cancer (Mahoney, 2010; Rana and Mahmood, 2010; Rosenwasser, 2010; Takeda and Maemura, 2010). Malnutrition, even as early as the developmental period, can also have adverse effect on clock function. Studies have shown maternal dietary protein restriction during pregnancy adversely affected the quality of the sleep–wake cycle and locomotor activity rhythm in rat offspring (Datta et al., 2000; Duran et al., 2005). Thus the developing fetal clock system is sensitive to the environment and physiology of the pregnant mother.

The gene and protein components of the endogenous molecular clock, and their interaction have been well characterized (Buhr and Takahashi, 2013; Dibner et al., 2010; Rosbash, 2009). However, it remains to be elucidated how the various environmental cues impact on clock function. One of the key techniques used to characterize the gene profiles of the endogenous molecular clock system within the SCN is quantitative reverse transcription polymerase chain reaction (qRT-PCR). However, the accuracy of such data is dependent on normalization by appropriate internal control reference genes, termed housekeeping genes (HKGs). Identifying stable control genes is important to ensure the validity of gene expression studies, as normalizing the gene of interest using the geometric mean of multiple internal control genes increases the quality of the data (Vandesompele et al., 2002). This controls for variables such as the amount or quality of starting material, enzymatic efficiencies, and differences between tissues or cells in overall transcriptional activity (Vandesompele et al., 2002).

We and others have previously shown that the expression level of these endogenous HKGs varies according to tissue type (Bruce et al., 2012; Hsiao et al., 2001; Sadek et al., 2012a, 2012b) and developmental stage (Sellayah et al., 2008; Warrington et al., 2000). They are constitutively expressed in the tissue and as they mediate basic cellular function were thought unlikely to vary due to the experimental conditions being investigated. However, many HKGs commonly used as internal controls, although stably expressed under physiological conditions, can vary significantly in their expression under certain experimental conditions (Radonic et al., 2004; Suzuki et al., 2000). For example in rat brain, the expression of the HKGs 18S rRNA and cyclophilin B (*CypB*) was altered in the cortex and hippocampus following dietary restriction and dexamethasone treatment (Tanic et al., 2007). Other conditions such as hypoxia not only altered *CypB* but also the commonly used HKG glyceraldehyde 3-phostphate dehydrogenase (*Gapdh*) and beta actin (*B-Act*) (Zhong and Simons, 1999). It is therefore vital to establish the most stable HKGs in the tissue of interest when selecting internal controls under the experimental conditions being investigated.

In this study we investigated the expression of 10 classic HKG across the 24 h light–dark cycle in the SCN of mouse offspring exposed to normal chow or high fat nutrition during early development and in postnatal life. The HKGs *Gapdh*, *B-Act* and adenosine triphosphate synthase subunit (*Atp5b*) were found to be the most stably expressed genes in the SCN regardless of diet or time within the 24 h light–dark cycle and are therefore suitable internal controls. However SCN samples collected during the light and dark periods did show differences in expression and as such the timing of collection should be considered when carrying out qPCR studies.

2. Results

In SCN samples collected during the dark period, there was a significant decrease in the expression of *Atp5b* (4.5-fold, $p=0.009$), *B-Act* (3.6-fold, $p=0.02$), *Cyc1* (3.9-fold, $p=0.03$), *Eif4a2* (5.4-fold, $p=0.001$), *Ubc* (3.4-fold, $p=0.01$), *Gapdh* (4.7-fold, $p=0.04$), and *Ywhaz* (2.8-fold, $p=0.04$), and a trend towards reduction in *Sdha* (2.8-fold, $p=0.08$) and *B2M* (1.5-fold, $p=0.06$) compared to samples collected during the light period (Fig. 1). There was no significant effect of diet on gene expression (Fig. 1).

The 10 genes were ordered according to stability (Fig. 2) and the number of genes required for normalization determined (Fig. 3). The pair-wise variation with the sequential addition of each reference gene indicated that two genes are sufficient as internal controls as the addition of a third gene gave a V score below 0.15 indicating that the additional gene had no significant contribution to the normalization factor (Fig. 3). The housekeeping genes *Gapdh*, *B-Act* and *Atp5b* showed the highest stability with all SCN samples analyzed together (Fig. 2) and when samples in the dark period were analyzed separately. For samples in the light period, *Sdha* replaced *Atp5b* as the third most stable gene.

3. Discussion

Accurate qPCR data is dependent on normalization to counteract sample and experimental variation. The most common approach is to normalize mRNA data to the expression of an