Mood-stabilizers differentially affect housekeeping gene expression in human cells

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Abstract

Recent studies have revealed that antidepressants affect the expression of constitutively expressed “housekeeping genes” commonly used as normalizing reference genes in quantitative polymerase chain reaction (qPCR) experiments. There has yet to be an investigation however on the effects of mood-stabilizers on housekeeping gene stability. The current study utilized lymphoblastoid cell lines (LCLs) derived from patients with mood disorders to investigate the effects of a range of doses of lithium (0, 1, 2 and 5 mM) and sodium valproate (0, 0.06, 0.03 and 0.6 mM) on the stability of 12 housekeeping genes. RNA was extracted from LCLs and qPCR was used to generate cycle threshold (C_t) values which were input into RefFinder analyses. The study revealed drug-specific effects on housekeeping gene stability. The most stable housekeeping genes in LCLs treated: acutely with sodium valproate were *ACTB* and *RPL13A*; acutely with lithium were *GAPDH* and *ATP5B*; chronically with lithium were *ATP5B* and *CYC1*. The stability of *GAPDH* and *B2M* were particularly affected by duration of lithium treatment. The study adds to a growing literature that the selection of appropriate housekeeping genes is important for the accurate normalization of target gene expression in experiments investigating the molecular effects of mood disorder pharmacotherapies. Copyright © 2014 John Wiley & Sons, Ltd.

Introduction

Mood-stabilizers such as lithium and sodium valproate are commonly prescribed pharmacotherapies used in the treatment of bipolar disorder. Despite being clinically prescribed, the mechanisms of action of each of these drugs are still relatively unknown (Shaldubina *et al.*, 2001). It is largely accepted however that gene expression

changes associated with these drug treatments likely play a role in their therapeutic effects (Sugawara *et al.*, 2010). Numerous studies using patient tissue samples, such as brain and blood, have attempted to unearth the molecular pharmacology of these mood-stabilizing drugs and how they confer their therapeutic effects (McQuillin *et al.*, 2007; Chetcuti *et al.*, 2006; Tsuang *et al.*, 2005). The quantitative polymerase chain reaction (qPCR) is the standard

method of investigating candidate gene expression changes and is used to validate large-scale microarray expression hits (VanGuilder *et al.*, 2008).

qPCR is a reaction in which a particular gene or region of DNA is amplified and detected in real-time. The relative quantification method of normalization is often employed for qPCR data and requires the subtraction of relatively stable reference genes' expression from the potentially more dynamic expression of the target gene (Livak and Schmittgen, 2001). As the reference genes used for normalization are subject to the same conditions as the target gene itself, it helps to control for variables such as RNA integrity and reverse transcription efficiency, as well as controlling for differences in the amount of starting material.

Most studies have used so-called "housekeeping" genes as the reference genes for normalization, as the products of these genes are essential for basal cell metabolism and so are assumed to be expressed at a constant and detectable level in all nucleated cell types during all developmental stages (Thellin *et al.*, 1999). Many of the commonly used housekeeping genes such as beta-actin (*ACTB*), glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*), 18S rRNA (18S) and beta-2-microglobulin (*B2M*) were first used in traditional qualitative or semi-quantitative methods because of their high expression levels in all cells (Hendriks-Balk *et al.*, 2007).

Recent research however suggests that housekeeping genes are not as stable as was originally thought, with evidence demonstrating different levels of stability in different cell types (Vandesompele *et al.*, 2002) and conditions (Hruz *et al.*, 2011). Previous studies have reported that antidepressants cause changes to the expression of housekeeping genes (Sugden *et al.*, 2010; Powell *et al.*, 2012). For instance, Sugden *et al.* (2010) investigated the effects of antidepressants on housekeeping gene expression in a mouse fibroblast cell line. It was revealed that antidepressants affect housekeeping gene expression stability with some genes (ATP synthase and cytochrome c1) showing greater levels of stability than others [Eukaryotic translation initiation factor 4A2 (*Eif4a2*)].

Subsequently, the selection of appropriate housekeeping genes is important for the accurate normalization of gene expression experiments investigating the molecular effects of pharmacotherapies for major depressive disorder. However, there is no evidence as to whether mood-stabilizers used to treat bipolar disorder also affect the expression of housekeeping genes. In this study we aimed to investigate the *in vitro* effects of the mood-stabilizers lithium and sodium valproate on the expression of a panel of candidate housekeeping genes using mood disorder patient-derived lymphoblastoid cell lines (LCLs) treated with these drugs.

We aimed to establish: the most appropriate reference genes for LCLs treated with each drug, the most inappropriate reference genes for LCLs treated with each drug, and the effects of drug treatment duration on housekeeping gene stability.

Materials and methods

Participants

Samples in this study were collected as part of the Depression Case Control (DeCC) Study. The clinical methodology used in the DeCC collection was described in detail previously (see Gaysina *et al.*, 2007). Briefly, subjects were identified from psychiatric clinics, hospitals, and general medical practices, and from volunteers responding to media advertisements. Only subjects of White European parentage were included. Subjects were over the age of 18 and had experienced two or more episodes of depression of at least moderate severity separated by at least two months of remission as defined by the Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition (DSM-IV) operational criteria, or the International Classification of Diseases, 10th Revision (ICD-10) operational criteria. Participants were excluded if they had schizophrenia or bipolar disorder or their depression was caused by a physical illness, medication, alcohol or substance abuse, if they were intravenous drug users or were related to someone already recruited for the study. The study was approved by the Joint South London and Maudsley NHS Trust Institute of Psychiatry Research Ethics Committee and informed written consent was obtained from all the participants at the time of sample collection. Ten milliliters of whole blood from each individual was sent to the Human Genetic Cell Bank at the European Collection of Cell Cultures (ECACC), during which immortalized Epstein-Barr virus (EBV)-transformed LCLs were generated. The current study utilized LCLs from five females diagnosed with major depressive disorder, with a mean age of 53.6 ± 11.7 years, and who had not previously taken any mood-stabilizing medications.

Cell culture

The LCL samples were supplied by ECACC as frozen ampoules containing 1 ml of cells at a density of approximately 2×10^7 cells/ml, in cell culture freezing medium [10% dimethyl sulfoxide (DMSO)]. Five cell lines were selected and grown in suspension in RPMI-1640 medium (Sigma-Aldrich, Poole, UK) supplemented with 10% foetal bovine serum, 2 mM L-glutamine, 100 units/ml penicillin, 0.1 mg/ml streptomycin and 0.05 mg/ml neomycin

at 37°C in a humidified atmosphere containing 5% carbon dioxide (CO₂) (see Supplementary Information, S1).

Drug treatment of LCLs

The lithium chloride drug (Sigma-Aldrich) dilution was prepared by diluting the supplied 8 M lithium chloride (LiCl) solution in RNase-free water to produce a sterile 1 M LiCl stock solution, and then further diluting the stock solution to 1, 2 and 5 mM for the treatment procedure. Sodium valproate (Sigma-Aldrich) was obtained in powder form and diluted in RNase-free water to a concentration of 3 mM. This stock solution was then diluted accordingly to concentrations of 0.06, 0.3 and 0.6 mM for the treatment procedure. A vehicle control dilution was also prepared using RNase-free water in serum-free supplemented growth medium.

Each of the five cell lines underwent an acute drug treatment as part of a four-stage culture protocol: (i) 72-hour growth phase, (ii) 24-hour serum-starve phase, (iii) 24-hour drug administration, and (iv) 24-hour recovery phase (for details see Supplementary Information, S2). After the final 24-hour recovery phase, cell pellets were obtained following centrifugation and were immediately stored at −80°C for RNA extraction.

Each of the five cell lines were also treated with a chronic seven-day dose of LiCl. This involved the same doses as with the acutely treated cells but instead utilized a two stage culture protocol: (i) 72-hour growth phase and a (ii) seven-day lithium administration phase (see Supplementary Information, S3 for details).

RNA extraction and cDNA synthesis

RNA extraction was performed using TRI reagent (Sigma-Aldrich). The purity and quantity of RNA was subsequently measured using the Nanodrop ND1000 (Thermoscientific, Wilmington, DE), which showed all samples had 260/280 ratios of between 1.8 and 2.1. The RNA integrity numbers (RINs) of samples were assessed using the Agilent 2100 Bioanalyzer (Agilent Technologies, Berkshire, UK) and all samples showed RINs > 9. The reverse transcription reaction was prepared using 1 µg of total RNA and the QuantiTect Reverse Transcription Kit (Qiagen, Crawley, UK). Complementary DNA (cDNA) synthesis was carried out in two steps: a genomic DNA (gDNA) wipe-out step, followed by reverse transcription step, as according to the manufacturer's protocol. Briefly, following gDNA removal, the samples were incubated for 15 minutes at 42°C with 1 µl Quantiscript Reverse Transcriptase, 5 × Quantiscript Reverse Transcriptase buffer, and 1 µl Reverse Transcriptase primer mix (oligo-dT and random primers). The reverse

transcriptase enzyme was subsequently inactivated at 95°C for three minutes. The cDNA samples generated were stored at −20°C prior to use in the qPCR experiments.

Quantitative polymerase chain reaction (qPCR)

The qPCR experiments were performed in 384-well plates using reagents from Primer Design (Southampton, UK). Reagents included Precision-R MasterMix and the human geNorm Kit (PrimerDesign) which included 12 pre-designed PerfectProbeTM fluorescent (FAM-labelled) primer/probe sets for 12 human housekeeping genes. No cycle threshold (C_t) values for the housekeeping gene *Eif4a2* were detected in our samples. This was due to a technical problem with the primer/probe set that had been supplied for this gene. Therefore the gene *Eif4a2* was excluded from the assays and from the final analyses. Details of the remaining 11 housekeeping genes are shown in Table 1 (see Supplementary Information, S4 for more detailed information on primers). The qPCR was set up according to the manufacturer's instructions, whereby each well on our 384-well plate contained a 20 µl qPCR reaction mixture consisting of: 5 µl cDNA, 4 µl RNase-free water, 1 µl of primer/probe mix and 10 µl of Precision-R MasterMix. qPCR assays were performed in duplicate to generate two technical replicates, and a negative control sample was also included for each reference gene.

The qPCR reactions were performed using the ABI Prism 7900HT Sequence Detection System (Applied Biosystems, California, USA). Thermal cycling conditions consisted of an enzyme activation stage (95°C for 10 minutes), followed by 50 cycles of a denaturation stage (95°C for 15 seconds) and hybridization and data collection stage (50°C for 30 seconds), and a final extension stage (72°C for 15 seconds). The software program SDS 2.1 (Applied Biosystems) generated C_t values from the data collected.

Statistical analysis

Average C_t values from our technical replicates were input into The RefFinder statistical analysis web-based tool (available from <http://www.leonxie.com/referencegene.php>). This tool was used for evaluating the stability of putative reference genes using an integrative weighted analysis which incorporates results from four already established analyses used for the selection of reference genes. These four already established statistical analyses for the selection of reference genes include: geNorm [as described in Vandesompele *et al.* (2002)], Normfinder [as described in Andersen *et al.* (2004)], Bestkeeper [as described in Pfaffl *et al.* (2004)] and the comparative ΔC_t method [as described in Silver *et al.* (2006)]. Based on

Table 1. A list of the 11 housekeeping genes included in the human geNorm kit, accession number and protein function

Accession number	Gene name	Protein function
NM 001101	Actin, beta (<i>ACTB</i>)	Cytoskeletal structural protein
NM 002046	Glyceraldehyde-3-phosphate dehydrogenase (<i>GAPDH</i>)	Glycolytic pathway enzyme
NM 021009	Ubiquitin C (<i>UBC</i>)	Protein modifier implicated in numerous functions
NM 004048	Beta-2-microglobulin (<i>B2M</i>)	Beta chain of major histocompatibility complex class I molecules
NM 003406	Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide (<i>YWHAZ</i>)	Adapter protein involved in mediating signal transduction
NM 012423	Ribosomal protein L13a (<i>RPL13A</i>)	Component of the 60S ribosomal subunit
NM 022551	18S ribosomal RNA (18S rRNA, <i>18S</i>)	Component of the 40S ribosomal subunit
NM 001916	Cytochrome c-1 (<i>CYC1</i>)	Heme-containing component of cytochrome b-c1 complex of the mitochondrial respiratory chain
NM 004168	Succinate dehydrogenase complex, subunit A, flavoprotein (<i>SDHA</i>)	Involved in complex II of the mitochondrial electron transport chain
NM 003286	Topoisomerase (DNA) I (<i>TOP1</i>)	Enzyme that controls and alters the topology of DNA during transcription
NM 001686	ATP synthase (<i>ATP5B</i>)	Subunit of mitochondrial ATP synthase, produces ATP from ADP using a proton gradient across the membrane

the rankings from each method, RefFinder assigns an appropriate weight to an individual gene and calculates the geometric mean of their weights for the overall final ranking.

geNorm analysis was also considered separately as it can additionally be used to calculate the optimal number of reference genes required for normalization purposes. It achieves this by calculating standard deviations (SDs) of log-transformed expression ratios for all housekeeping genes and then carrying out a pairwise comparison of the SD of a particular gene with each of the remaining other housekeeping genes. One by one, the least stable reference genes are excluded based on high *M* values (which represent expression stability scores), leaving at least two remaining genes that correspond to the most stably expressed genes. According to Vandesompele *et al.* (2002), a combination of two or more reference genes producing a *V* (variation measure) of $V < 0.15$ is sufficient for optimal normalization.

Results

Number of reference genes required for accurate normalization

The geNorm approach was used to establish the number of reference genes for accurate normalization. The analysis revealed that two reference genes were optimal, as three

reference genes would not increase accuracy above that obtained using two reference genes. The variation between the mean of the two most stable genes compared with that of the three most stable was $V = 0.05$ for acute lithium treated cells, $V = 0.04$ for chronic lithium treated cells, and $V = 0.04$ for acute sodium valproate treated cells; all of which are well below the threshold of $V < 0.15$ proposed by Vandesompele *et al.* (2002). The pairwise variation was relatively stable across all the comparisons, none of which exceeded the threshold value of 0.15 (see Supplementary Information, S5–S7). Nevertheless, the selection of the most stable housekeeping genes will likely increase the chances of detecting target gene expression differences of smaller magnitudes.

Acute treatment (24 hours) of LCLs with sodium valproate

The C_t values of 11 housekeeping genes in LCLs treated with the different concentrations of sodium valproate are shown in Figure 1. The lowest generated mean C_t value, and therefore the most highly expressed gene was *18S* (16.87 ± 0.19 SD). The highest mean C_t value and therefore the lowest expressing gene was *SDHA* (30.11 ± 0.67 SD). RefFinder analyses revealed that *ACTB* and *RPL13A* were the two most stable reference genes producing

Figure 1. qPCR cycle threshold values in 11 reference genes in LCLs treated with 0, 0.06, 0.3 and 0.06 mM sodium valproate for 24 hours. Expression levels are shown as median (lines), 25th percentile to the 75th percentile (boxes), and ranges (whiskers). The mean C_t values (white diamonds) and outliers (black triangles) are also indicated.

Figure 2. Bar chart showing RefFinder expression stability values (y-axis) for 11 reference genes (x-axis) in LCLs treated with 0, 0.06, 0.3 or 0.6 mM sodium valproate for 24 hours.

stability scores of 1.32 and 1.68, respectively. The two most unstable reference genes were *SDHA* and *CYC1* producing stability scores of 9.24 and 10.24, respectively, see Figure 2. Results from each of the individual analyses contributing to RefFinder results (geNorm, Normfinder, Bestkeeper and the comparative ΔC_t method) can be found in the Supplementary Information, S8.

Acute treatment (24 hours) of LCLs with lithium

The C_t values of 11 housekeeping genes in LCLs treated with different concentrations of lithium for 24 hours are shown in Figure 3. The lowest mean C_t value, and therefore most highly expressed gene was *18S* (18.79 ± 1.74 SD), whilst the highest mean C_t value, and lowest expressed gene was succinate dehydrogenase complex, subunit A,

Figure 3. qPCR cycle threshold values in 11 reference genes in LCLs treated with 0, 1, 2 or 5 mM lithium chloride for 24 hours. Expression levels are shown as median (lines), 25th percentile to the 75th percentile (boxes), and ranges (whiskers). The mean C_t values (white diamonds) and outliers (black triangles) are also indicated.

Figure 4. Bar chart showing RefFinder expression stability values (y-axis) for 11 reference genes (x-axis) in LCLs treated with 0, 1, 2 or 5 mM lithium chloride for 24 hours.

flavoprotein (*SDHA*) (27.56 ± 1.45 SD). RefFinder analyses revealed that *GAPDH* and *ATP5B* were the two most stable reference genes producing stability scores of 2 and 2.63, respectively. The two most unstable reference genes were *SDHA* and *18S* producing stability scores of 9.49 and 11, respectively, see Figure 4. Results from each of the individual analyses contributing to RefFinder results (geNorm, Normfinder, Bestkeeper and the comparative ΔC_t method) can be found in the Supplementary Information, S9.

Chronic treatment (seven days) of LCLs with lithium

The C_t values of 11 housekeeping genes in LCLs treated with different concentrations of lithium for seven days are shown in Figure 5. The lowest mean C_t value, and therefore most highly expressed gene was *18S* (18.26 ± 0.60 SD), whilst the highest mean C_t value, and lowest expressed gene was *SDHA* (29.28 ± 0.53 SD). RefFinder analyses revealed that *ATP5B* and *CYC1* were the two most stable reference genes producing stability scores of 1.78 and 2.63, respectively. The two most

Figure 5. qPCR cycle threshold values in 11 reference genes in LCLs treated with 0, 1, 2 or 5 mM lithium chloride for seven days. Expression levels are shown as median (lines), 25th percentile to the 75th percentile (boxes), and ranges (whiskers). The mean C_t values (white diamonds) and outliers (black triangles) are also indicated.

Figure 6. Bar chart showing RefFinder expression stability values (y-axis) for 11 reference genes (x-axis) in LCLs treated with 0, 1, 2 or 5 mM lithium chloride for seven days.

unstable reference genes were *SDHA* and *ACTB* producing stability scores of 10 and 11, respectively, see Figure 6. Results from each of the individual analyses contributing to RefFinder results (geNorm, Normfinder, Bestkeeper and the comparative ΔC_t method) can be found in the Supplementary Information, S10. A comparison of the stability values of the housekeeping genes in LCLs following acute and chronic lithium treatment are shown in Figure 7.

Discussion

Recent studies investigating the effects of antidepressants *in vitro* and *ex vivo* revealed that housekeeping gene expression, which was once believed to be “stable” across conditions, in fact shows expression variability (Sugden *et al.*, 2010; Powell *et al.*, 2012). This in turn has consequences for studies investigating the gene expression effects of mood disorder pharmacotherapies using qPCR experiments and the relative quantification method of

Figure 7. Comparison of the 11 reference gene expression profiles for LCLs treated with 0, 1, 2 or 5 mM lithium chloride for 24 hours (grey line) or for seven days (black line). Gene names are shown on the x-axis and RefFinder stability values are marked on the y-axis.

normalization. The current study aimed to investigate the effects of the mood-stabilizers lithium and sodium valproate, which are used to treat bipolar disorder, on housekeeping gene expression. This study investigated the effects of acute drug treatments (sodium valproate, lithium) and a chronic drug treatment (lithium) at a variety of doses on the expression of a panel of 11 housekeeping genes. The drug dose ranges chosen incorporates doses that are believed to be therapeutic. This is based on serum concentrations of lithium found in bipolar disorder patients (approximately 1 mM) (Taylor *et al.*, 2007), and on the dose required for sodium valproate to demonstrate neuroprotective effects (approximately 0.1 mM) (Biermann *et al.*, 2011). The current study used an *in vitro* experimental design carried out using LCLs derived from mood disorder patients. LCLs have previously been shown to have gene expression profiles which correlate highly with levels of gene expression in non-transformed lymphocytes and whole blood collected from psychiatric patients (Rollins *et al.*, 2010). Consequently, these cell lines were specifically selected from mood disorder patients with the aim of creating the best *proxy* model for investigating the gene expression effects of mood-stabilizers that may occur *in vivo* in mood disorder patients.

The study revealed that all housekeeping genes were expressed at acceptably detectable levels under drug treatment conditions (i.e. $C_t < 37$) in LCLs producing a broad range of C_t values (see Figures 1, 3 and 5). Across all drug groups, 18S was the most highly expressed housekeeping gene, whereas SDHA was the lowest expressing gene. Both mood-stabilizers were found to affect the stability of housekeeping gene expression and they did so differentially in a

drug-specific manner. geNorm analyses revealed that two housekeeping genes were sufficient for optimal normalization of target genes in all drug treatment groups.

In the LCLs treated acutely with sodium valproate, the two most stable housekeeping genes according to the RefFinder analyses were ACTB and RPL13A (see Figure 2). ACTB, a gene encoding a cytoskeletal structural protein, has previously been used as a normalizing reference gene in mice treated with sodium valproate (Wu *et al.*, 2010) and has been shown to be one of the most stable housekeeping genes in studies investigating the effects of valproate on forskolin-stimulated human adrenal carcinoma (H295R) cells (von Krogh *et al.*, 2010). RPL13A, a component of the 60S ribosomal subunit, has been shown to be one of the most stable housekeeping genes in a rat model of cerebral ischemia (Tian *et al.*, 2006), but has not been previously used for normalization purposes in cells treated with sodium valproate. The housekeeping genes SDHA and CYC1 showed the highest expression variability according to RefFinder analyses, and thus would be considered unsuitable as reference genes in LCLs exposed acutely to sodium valproate (see Figure 2).

In the LCLs acutely treated with lithium, the two most stable housekeeping genes according to RefFinder were GAPDH and ATP5B (see Figure 4). GAPDH, a gene encoding a glycolytic pathway enzyme has previously been shown to demonstrate stable gene expression in human epithelial cells treated with lithium (Nemeth *et al.*, 2002) and has also previously been used for normalization in human placental cells treated with the drug (Roberts *et al.*, 2007). ATP5B, a gene which encodes a protein involved in catalysing ATP formation has previously been shown

to be one of the most stable genes in cells treated with antidepressants (Sugden *et al.*, 2010). *ATB5B*, was further shown to be the most stable reference gene in LCLs treated with lithium chronically along with *CYC1* (see Figure 6). The greatest variation in expression and therefore worst reference genes in LCLs treated acutely with lithium were observed in the genes *SDHA* and *18S* (see Figure 4 and Table 1), and in *SDHA* and *ACTB* in LCLs treated chronically with lithium.

Previous research on the effects of antidepressants revealed that duration of drug treatment had little effect on housekeeping gene expression stability (Sugden *et al.*, 2010). Here, we tested whether duration of treatment with a mood-stabilizer might affect housekeeping gene expression. We observed the effects of a chronic (seven day) administration of lithium on housekeeping gene expression in LCLs, and compared it to an acute 24 hour treatment of lithium. The stability of genes in LCLs treated chronically showed a similar trend to the relative stability of genes in LCLs treated acutely. However, there were two genes which were key exceptions. In the chronically treated cells, *GAPDH* became less stable and *B2M* became more stable (see Figure 7). Subsequently, *GAPDH* may be the most stable reference gene in LCLs treated acutely with lithium, but it is less suitable for LCLs treated chronically with the drug. If comparisons were to be drawn across different drug duration groups, *ATP5B* and *UBC* would in fact be considered two of the most stable reference genes (see Figure 7). These results consequently suggest that careful consideration is needed for the selection of reference genes not only for mood-stabilizer type but also for treatment duration.

To conclude, this is the first study to investigate the differential effects of mood-stabilizers on housekeeping gene expression in human cells from mood disorder patients. The study revealed that lithium and sodium valproate caused drug-specific effects on housekeeping gene expression stability, with lithium treatment duration also having

some influences on housekeeping gene stability, particularly in the genes *GAPDH* and *B2M*. *ACTB* and *RPL13A* were the two most stable genes in LCLs treated acutely with sodium valproate, whereas *GAPDH* and *ATP5B* were the two most stable genes in LCLs treated acutely with lithium, and *ATB5B* and *CYC1* were the two most stable genes in LCLs treated chronically with lithium. *SDHA* was amongst the most variably expressed housekeeping genes across all drug groups and as such may be considered an unsuitable reference gene in *in vitro* experiments investigating the effects of mood-stabilizers. The use of mood-disorder patient derived LCLs in this study arguably means results from this *in vitro* experiment might not only extrapolate to other *in vitro* studies investigating the effects of mood-stabilizers but also to *ex vivo* studies investigating gene expression in lymphocytes extracted from mood disorder patients; although this would need to be confirmed in future studies. The study provides further evidence to a growing literature that the selection of appropriate housekeeping genes is important for the accurate normalization of target gene expression in experiments investigating the molecular effects of mood disorder pharmacotherapies.

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Declaration of interest statement

Powell, Powell-Smith, Haddley, Quinn, Schalkwyk, and D'Souza report no competing interests. McGuffin and Farmer have received consultancy fees and honoraria for participating in expert panels from pharmaceutical companies, including Lundbeck and GlaxoSmithKline.

References

- Andersen C.L., Jensen J.L., Ørntoft T.F. (2004) Normalization of real-time quantitative reverse transcription-PCR data: a model-based variance estimation approach to identify genes suited for normalization, applied to bladder and colon cancer data sets. *Cancer Research*, **64**(15), 5245–5250.
- Biermann J., Boyle J., Pielen A., Lagrèze W.A. (2011) Histone deacetylase inhibitors sodium butyrate and valproic acid delay spontaneous cell death in purified rat retinal ganglion cells. *Molecular Vision*, **17**L, 395–403.
- Chetcuti A., Adams L.J., Mitchell P.B., Schofield P. R. (2006) Altered gene expression in mice treated with the mood stabilizer sodium valproate. *International Journal of Neuropsychopharmacology*, **9**(3), 267–276.
- Gaysina D., Cohen S., Craddock N., Farmer A., Hoda F., Korszun A., Owen M.J., Craig I.W., McGuffin P. (2007) No association with the 5,10-methylenetetrahydrofolate reductase gene and major depressive disorder: results of the Depression Case Control (DeCC) study and a meta-analysis. *American Journal of Medical Genetics Part B*, **147B**(6), 699–706.
- Hendriks-Balk M.C., Michel M.C., Alewijnse A.E. (2007) Pitfalls in the normalization of real-time polymerase chain reaction data. *Basic Research in Cardiology*, **102**(3), 195–197.
- Hruz T., Markus W., Docquier M., Pfaffl M.W., Masanetz S., Borghil L., Verbrughe P., Kalaydjieva L., Bleuler S., Laule O., Descombes P., Gruissem W., Zimmermann P. (2011) RefGenes: identification of reliable

- and condition specific reference genes for RT-qPCR data normalization. *BMC Genomics*, **12**, 156.
- von Krogh K., Harjen H., Almås C., Zimmer K.E., Dahl E., Olsaker I., Taubøll E., Ropstad E., Verhaegen S. (2010) The effect of valproate and levetiracetam on steroidogenesis in forskolin-stimulated H295R cells. *Epilepsia*, **51**(11), 2280–2288.
- Livak K.J., Schmittgen T.D. (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2(-delta delta C(T)) method. *Methods*, **25**(4), 402–408.
- McQuillin A., Rizig M., Gurling H.M. (2007) A microarray gene expression study of the molecular pharmacology of lithium carbonate on mouse brain mRNA to understand the neurobiology of mood stabilization and treatment of bipolar affective disorder. *Pharmacogenetics and Genomics*, **17**(8), 605–617.
- Nemeth Z.H., Deitch E.A., Szabo C., Fekete Z., Hauser C.J., Hasko, G. (2002) Lithium induces NF-κB activation and interleukin-8 production in human intestinal epithelial cells. *Journal of Biological Chemistry*, **277**, 7713–7719.
- Pfaffl M.W., Tichopad A., Prgomet C., Neuvians T. P. (2004) Determination of stable housekeeping genes, differentially regulated target genes and sample integrity: BestKeeper – Excel-based tool using pair-wise correlations. *Biotechnology Letters*, **26**, 509–515.
- Powell T.R., Schalkwyk L.C., Heffernan A.L., Breen G., Lawrence T., Price T., Farmer A.E., Aitchison K.J., Craig I.W., Danese A., Lewis C., McGuffin P., Uher R., Tansey K.E., D'Souza U.M. (2012) Tumor necrosis factor and its targets in the inflammatory cytokine pathway are identified as putative transcriptomic biomarkers for escitalopram response. *European Neuropsychopharmacology*, **23**(9), 1105–1114.
- Roberts J., Scott A.C., Howard M.R., Breen G., Bubb V.J., Klenova E., Quinn J.P. (2007) Differential regulation of the serotonin transporter gene by lithium is mediated by transcription factors, CCCTC binding protein and Y-box binding protein 1, through polymorphic intron 2 variable number tandem repeat. *Journal of Neuroscience*, **27**(11), 2793–2801.
- Rollins B., Martin M.V., Morgan L., Vawter M.P. (2010) Analysis of whole genome biomarker expression in blood and brain. *American Journal of Medical Genetics Part B*, **153B**(4), 919–936.
- Shaldubina A., Agam G., Belmaker R.H. (2001) The mechanism of lithium action: state of the art, ten years later. *Progress in Neuro-Psychopharmacology and Biological Psychiatry*, **25**(4), 855–866.
- Silver N., Best S., Jiang J., Thein S.L. (2006) Selection of housekeeping genes for gene expression studies in human reticulocytes using real-time PCR. *BMC Molecular Biology*, **7**, 33.
- Sugawara H., Iwamoto K., Bundo M., Ishiwata M., Ueda J., Kakiuchi C., Ishigooka J., Kato T. (2010) Effect of mood stabilizers on gene expression in lymphoblastoid cells. *Journal of Neural Transmission*, **117**(2), 155–164.
- Sugden K., Pariante C.M., McGuffin P., Aitchison K.J., D'Souza U.M. (2010) Housekeeping gene expression is affected by antidepressant treatment in a mouse fibroblast cell line. *Journal of Psychopharmacology*, **24**(8), 1253–1259.
- Taylor D., Kerwin R., Paton C. (2007) The Maudsley Prescribing Guidelines, 9th edition. London: Informa Healthcare.
- Thellin O., Zorzi W., Lakaye B., De Borman B., Coumans B., Hennen G., Grisar T., Igout A., Heinen E. (1999) Housekeeping genes as internal standards: use and limits. *Journal of Biotechnology*, **75**(2-3), 291–295.
- Tian Y.F., Zhang P.B., Xiao X.L., Zhang J.S., Zhao J.J., Kang Q.Y., Chen X.L., Qiu F., Liu Y. (2006) The quantification of ADAMTS expression in an animal model of cerebral ischemia using real-time PCR. *Acta Anaesthesiologica Scandinavica* **51**(2), 158–164.
- Tsuang M.T., Nossova N., Yager T., Tsuang M.M., Guo S.C., Shyu K.G., Glatt S.J., Liew C.C. (2005) Assessing the validity of blood-based gene expression profiles for the classification of schizophrenia and bipolar disorder: a preliminary report. *American Journal of Medical Genetics Part B*, **133B**(1), 1–5.
- Vandesompele J., Preter K.D., Pattyn F., Poppe B., Van Roy N., De Paepe A., Speleman F. (2002) Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biology*, **3**, RESEARCH0034.
- VanGuilder H.D., Vrana K.E., Freeman W.M. (2008) Twenty-five years of quantitative PCR for gene expression analysis. *BioTechniques*, **44**(5), 619–626.
- Wu G., Nan C., Rollo J.C., Huang Z., Tien J. (2010) Sodium valproate-induced congenital cardiac abnormalities in mice are associated with the inhibition of histone deacetylase. *Journal of Biomedical Science*, **17**, 16.

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