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Gene Expression Biomarkers for Neurobehavioral Impairment From Total Sleep Deprivation

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16. Abstract

Sleep deprivation (SD) poses health and safety risks to the general public, and can be a particular danger to workers in safety-critical roles. However, the ability to identify operationally dangerous levels of sleep loss is hindered by the lack of a conclusive test. This study employs microarray analyses toward developing gene expression biomarkers diagnostic of not only SD, but more importantly, the phenotype of neurobehavioral impairment from sleep loss. Healthy adult volunteers were recruited to a sleep laboratory for seven consecutive days, six nights. After two Baseline days of 10 h time in bed (TIB), 11 subjects underwent an Experimental phase of 62 h of continued wakefulness, followed by two Recovery nights of 10 h TIB. Another six subjects underwent a well-rested Control condition of 10 h TIB for all six nights. Blood draws were taken for measuring gene expression on days two, four, and six at 4 h intervals from 08:00 to 20:00 h, corresponding to 12 timepoints across one Baseline, one Experimental, and one Recovery day. Altogether 212 genes changed expression in response to the SD Treatment, i.e., the difference between SD and Control subjects. Most genes were down-regulated during SD. Also, 28 genes were associated with neurobehavioral deficits as measured by the Psychomotor Vigilance Test (PVT). The changes found in many of both the Treatment and PVT genes support previous findings associating SD with the immune response and ion signaling. Additionally, novel biomarkers are reported such as the Speedy/RINGO family of cell cycle regulators. This study serves as an important first step toward gene expression biomarker discovery for neurobehavioral impairment from sleep deprivation.

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INTRODUCTION

Sleep plays a key role in health, performance, and cognition (SPIEGEL *et al.* 1999; VAN DONGEN *et al.* 2003; FRANZEN *et al.* 2008; KILLGORE 2010; LAPOSKY *et al.* 2016). Yet sleep deficiencies, be they from mistimed sleep, inadequate sleep duration, or sleep disorders, are widespread (LAPOSKY *et al.* 2016). Annual losses in workplace productivity within the United States are estimated at over 60 billion dollars from insomnia alone (KESSLER *et al.* 2011). Psychological and physiological impairments from poor sleep pose a risk of injury and even mortality in fields with safety-critical roles. Over a 12 year period, approximately 20% of all major accidents investigated by the National Transportation Safety Board, including approximately 40% of highway and 23% of aviation accidents, were associated with fatigue (MARCUS and ROSEKIND 2016). Fatigue can be a nebulous concept to define let alone quantify, although it has been described in terms of both subjective (a feeling of exhaustion, tiredness, and low energy) and objective (neurobehavioral or physical impairments) components (SHEN *et al.* 2006). Causes of fatigue include high workload, stress, and sleep disorders; however, for this study the focus was acute total sleep deprivation (SD).

A wide range of neurobehavioral tests have demonstrated assorted forms of performance deficits from sleep loss, including impairment of learning and responses to feedback in decision making (KILLGORE 2010; LIM and DINGES 2010; WHITNEY *et al.* 2015). Since its introduction over 30 years ago, the Psychomotor Vigilance Test (PVT) is one of the most commonly applied assays (DINGES and POWELL 1985; LIM and DINGES 2008). This test assays stimulus-response time, with failure to respond within ~500 ms recorded as a lapse. Sleep deprivation is associated with increased variability in stimulus-response times, and more lapses, on the PVT (DORAN *et al.* 2001). For a real-world example, more time sleeping within 24 h of the duty start time was associated with faster PVT stimulus responses in pilots (GANDER *et al.* 2015).

Besides these neurobehavioral tests, efforts have been made to identify biomarkers such as differentially expressed genes or metabolites affected by sleep disruption (PELLEGRINO *et al.* 2012; DAVIES *et al.* 2014; ARCHER and OSTER 2015; GOEL 2015; WELJIE *et al.* 2015; MULLINGTON *et al.* 2016). Humans are known to differ in their sensitivity to sleep loss (VAN DONGEN *et al.* 2004; VAN DONGEN *et al.* 2012; PATANAIK *et al.* 2015), and recent work also has sought to identify biomarkers distinguishing individuals as susceptible or resistant to sleep deprivation (RUPP *et al.* 2013; ARNARDOTTIR *et al.* 2014; GOEL 2015; SATTERFIELD *et al.* 2015). Yet surprisingly little effort has been made to synthesize such biomarker research with results from neurobehavioral assays. Such an integrated approach can focus biomarker discovery on the scientifically and operationally relevant phenotype of impairment from SD, and may enhance fundamental understanding of how SD affects neurobehavioral performance at the molecular level.

This study tests the hypothesis that gene expression responds to total SD, and can be related to neurobehavioral impairment measured by PVT lapses. The approach was one of biomarker discovery, *sensu* (MULLINGTON *et al.* 2016). Healthy human adults were recruited to the Sleep and Performance

Research Center at Washington State University and acclimated with two nights of Baseline sleep for 10 h time-in-bed (TIB). Then subjects in the SD group underwent 62 h continuous wakefulness during an Experimental phase, followed by two Recovery nights of 10 h sleep opportunity. In contrast, individuals in the Control (C) group received 10 h TIB all six nights. Neurobehavioral impairment was assessed with the PVT, and blood was drawn for whole-transcriptome microarrays. Although gene expression may vary across fluid and tissue types, concordance as high as 80% has been reported between the transcriptome of blood and major tissues (LIEW *et al.* 2006; SUNDE 2010). A total of 212 genes were differentially expressed in response to Treatment, defined as a difference between SD and C subjects during the Experimental or Recovery phase. Also, the expression of 28 genes was associated with PVT lapses. Biological pathways and functions of the differentially expressed genes are discussed, as well as their utility as candidates for an operational sleep deprivation biomarker panel.

MATERIALS AND METHODS

Sample Collection and Neurobehavioral Data

Study design and population demographics, including subjects' prior sleep history, have been reported previously (WHITNEY *et al.* 2015). Ribonucleic acid extracted from blood was used to assess human transcriptomic changes during total SD. All individuals provided written informed consent, and Institutional Review Board approval was obtained at both the Civil Aerospace Medical Institute (CAMI) of the Federal Aviation Administration, and Washington State University (WSU).

In brief, blood samples were obtained from 17 healthy adults (ages 22–37, 7 females). Subjects remained in the sleep laboratory at the Sleep and Performance Research Center (WSU, Spokane, WA) for six consecutive nights. A two-night Baseline phase for acclimation was followed by a two-night Experimental phase, and the study concluded with two Recovery nights. During both the Baseline and Recovery phases all subjects received 10 h TIB each night (22:00–08:00 h). For the Experimental phase, 11 randomly selected subjects underwent SD, consisting of 62 h of continued wakefulness. The remaining 6 C subjects received the usual 10 h TIB nightly. Blood samples were collected with an intravenous catheter every 4 h during time awake on days two, four, and six (**Fig. 1**), corresponding to one day each during the Baseline, Experimental, and Recovery phases. At each of the 12 timepoints (**Fig. 1**, **Suppl. Table 1**), 2.5 mL blood was collected in a PAXgene^{TM} Blood RNA tube (PN 762165, PreAnalytiX GmbH, Hombrechtikon CH) and stored at approximately -20°C overnight. The following day, tubes were transferred to -80°C until shipment on dry ice to the CAMI Functional Genomics laboratory, where they were immediately returned to -80°C until RNA extraction. Of 204 possible samples (12 timepoints, 17 subjects), five blood draws were unsuccessful, resulting in 199 RNA extractions. Blood was not able to be drawn from two SD subjects at 08:00 h and a third SD subject at 20:00 h during the second day (Baseline), and from a fourth SD subject at 16:00 h on day six (Recovery). Likewise phlebotomists were unable to collect blood from one C subject at 16:00 h on day four **(Suppl. Table 1)**.

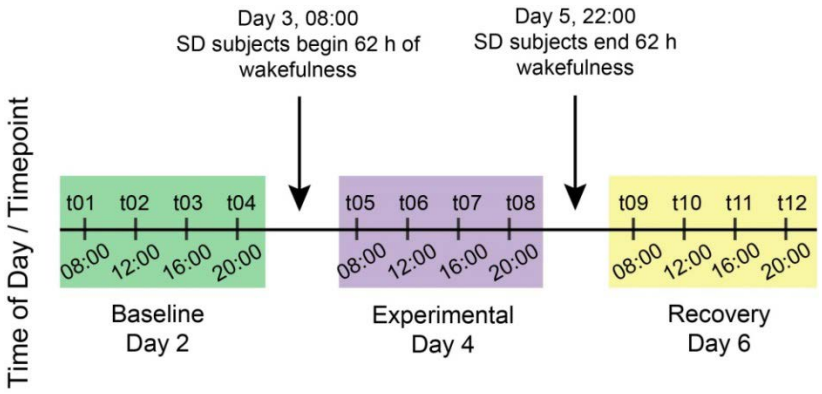

Figure 1. Overview of the study design, consisting of seven days, six nights in the sleep laboratory. During the Baseline and Recovery phases, all 17 subjects received 10 h TIB each night. During the Experimental phase, the 11 SD persons underwent a 62 h period of continued wakefulness, whereas the 6 C subjects continued to have 10 h TIB each night. Vertical lines indicate the 12 timepoints with blood collection and PVT data as analyzed here.

As previously described (WHITNEY *et al.* 2015), subjects were presented with the PVT for 10 min at 2 h intervals during scheduled wakefulness. However, for the present report only the test every 4th h corresponding to timing of blood draws was utilized (**Fig. 1**). Subjects were asked to immediately press a button upon observing a visual cue on a computer screen, which appeared at random 2–10 s intervals. Stimulus-response times >500 ms were recorded as a lapse, and data were recorded as the number of lapses per test bout. Significant differences in PVT over time and between conditions were tested with generalized linear models using Poisson distribution and log link with PVT as the response variable, and progressively adding as predictors Treatment (SD or C), Phase, Time of Day, and their interaction terms. In all models a random intercept was included for subject; also, a random term for observation was included to correct for overdispersion as in (ELSTON *et al.* 2001). Models with different predictors were compared using P-values from Maximum Likelihood tests, as well as Akaike Information Criterion (AIC) and Bayesian Information Criterion (BIC) scores. Models were constructed in R v. 3.2.1 using the glmer function in the lme4 v. 1.1-11 package (BATES *et al.* 2015), with the "nlminb" optimizer.

RNA Isolation

Samples in PAXgene^{TM} tubes were thawed at room temperature with slow rotation overnight. Tubes were centrifuged 10 min at 4000 x g in a swinging bucket rotor. The pellet was washed by resuspension in 4 mL RNase-free water and centrifugation for 10 min at 4000 x g. Total RNA from the pellet was isolated with a QIAcube robotic workstation (PN9001292 Qiagen Inc., Valencia CA), using reagents from the PAXgene^{TM} Blood miRNA Kit (PN763134, PreAnalytiX) and the directions found in Appendix C of the accompanying Kit Handbook, version 05/2009. The QIAcube protocols PAXgene^{TM} Blood miRNA Part A and Part B (www.qiagen.com/MyQIAcube) were used. Following isolation, aliquots were used for quantification on a NanoDrop 1000 Spectrophotometer (ThermoFisher), and quality assessment on an Agilent 2100 Bioanalyzer® with the RNA 6000 Nano kit (Agilent Technologies, PN 5067-1511).

Microarray Probe Preparation and Hybridization

Total RNA samples were randomized and aliquots were normalized to 20 ng/µl. Fifty nanograms of each sample were spiked with 2 µL of 3' amplification controls (Affymetrix, PN 900454). Reverse transcription was performed with the Ovation Pico WTA System V2 kit (PN3302, NuGEN Inc., San Carlos, USA) for generation of the amplified cDNA product. Concentration of the amplified cDNA was determined with the NanoDrop as above. Fragmentation and biotin labeling was performed on 5 µg amplification product using the Encore® Biotin module (PN4200, NuGEN Inc.), according to the manufacturer's protocol.

Hybridization cocktail including Affymetrix hybridization controls (PN900454) was prepared according to Appendix VI of the manual NuGEN Encore® Biotin Module for Affymetrix 169 format chips. GeneChip Human Gene 1.0 ST arrays (Affymetrix PN902113) were prewet with 90 µL prewarmed prehybridization solution for 10 min at 45°C with 60 rpm. The prehybridization solution was replaced with 90 µL of hybridization cocktail followed by 18 h incubation at 45°C with 60 rpm. The Affymetrix Wash and Stain kit reagents (PN900720-C) and fluidics protocol FS450-00007 were used with the GeneChip Fluidics Station 450 (PN 00-0079, Affymetrix) for post-hybridization chip processing and scanned on an Affymetrix Scanner (PN00-0073) with the G7 upgrade.

Differential Gene Expression: Treatment and PVT effect

Transcript Cluster-level expression values were derived by background correction, quantile normalization, and median polish summarization with the RMA algorithm (IRIZARRY *et al.* 2003) in the R/oligo package v. 1.32.0 (CARVALHO and IRIZARRY 2010). Data were filtered for low expression, only retaining a Transcript Cluster if at least 6 samples had $\log_2$ expression >6, corresponding to the median (and mean) expression of the antigenomic Transcript Clusters. Array data quality was assessed with hierarchical clustering and tools in R/ arrayQualityMetrics v. 3.24.0 (KAUFFMANN *et al.* 2009). Six arrays from six different SD subjects were considered outliers: one at noon and one at 20:00 h on day four (Experimental), one at noon and one at 20:00 h on day six (Recovery), and two at 16:00 h on day six. In tests of differential gene expression, outliers were included but de-emphasized using the internal array weighting function of limma (RITCHIE *et al.* 2015). For other analyses without an internal weighting function (WGCNA, MFuzz, and RIF; see below), a separate RMA expression file was created omitting these six outliers altogether.

Tests for differential expression were conducted for biomarker discovery with R/limma v. 3.24.15 using linear models (RITCHIE *et al.* 2015). All models included array weights, a random effect for subject (duplicateCorrelation function), and a term to account for differences attributable to Time of Day. Visual examination of the PVT data suggested that three of the 11 SD subjects were fatigue resistant, at least at the neurobehavioral level (**Suppl. Fig. 1**), and these were excluded from the RMA expression file for Treatment effect analysis. Thus, the Treatment effect analysis investigated the difference in gene expression between 8 SD and 6 C subjects at Baseline, at Experimental, and at the Recovery phase. The Treatment list was defined as Transcript Clusters with a significant difference between C and SD persons at the Experimental or Recovery phase (FDR <0.05), but not at Baseline. Plots of Transcript Clusters were reviewed to ensure mean $\log_2$ expression ±1 SE overlapped between C and SD subjects at all four Baseline timepoints, when no differences are expected. If plots showed separation in expression between C and SD at Baseline, the Transcript Cluster was discarded from the final Treatment list to reduce false positive discoveries.

Similarly, linear models were applied in limma to test for a significant relation of PVT lapses to gene expression. Here, data for all 11 SD and 6 C subjects were included. Besides the subject effect and PVT lapses, the model contained terms for Treatment (SD or C) and Time of Day. Because discovering a biomarker panel for neurobehavioral impairment from sleep deprivation was the primary aim, it was important to avoid missing candidate biomarkers in this first screening study. Hence the threshold for significance was relaxed from FDR <0.05 to FDR <0.10. As before, expression was plotted for SD and C subjects, and Transcript Clusters were eliminated from the PVT list if the mean $\log_2$ expression ±1 SE showed separation at any Baseline timepoint.

Co-expression and Temporal Networks

Weighted Gene Co-expression Network Analysis was performed using R/WGCNA v. 1.47 and data from all 17 subjects. This approach has been described in detail by Langfelder and Horvath (2008). Essentially the analysis serves to group genes based on similarity of expression across samples (n=193; 199 successful blood draws minus 6 outliers). All Transcript Clusters passing the low-expression filter were included, not just those differentially expressed, for a complementary approach to the limma models above. Signed networks were constructed, allowing for positive or inverse relationships based on Pearson correlations among genes. Using internal functions plotting scale free topology, a soft power threshold of 13 was selected, and the minimum number of Transcript Clusters per group was set to 30. Each group of co-expressed Transcript Clusters was termed a module, which the package designated by a color. Pearson correlations were computed separately between each module's eigengene and three variables: PVT lapses, Treatment, and Time of Day. The eigengene was a representative metric of the expression profile of Transcript Clusters in the module (LANGFELDER and HORVATH 2008). The WGCNA software also allowed identification of the top hub Transcript Cluster for each module, namely, the most highly connected Transcript Cluster within the module. Here, connectivity was based on the correlation of expression among genes (LANGFELDER and HORVATH 2008).

A second temporal clustering approach was performed using R/Mfuzz v. 2.28.0 (KUMAR and FUTSCHIK 2007). This analysis was not designed to test for treatment differences, but rather excels at portraying time series. Hence the three fatigue resistant individuals and the Controls were omitted, and Mfuzz was used to cluster and plot the mean $\log_2$ expression of the 8 SD subjects across the 12 timepoints. Expression values for the differentially expressed Treatment effect Transcript Clusters were z-transformed and then clustered across timepoints using fuzzy c-means clustering. An internal function was used to select the fuzzification parameter (m=1.33). A combination of internal functions and plotting was used to decide on the number of clusters, which was set to three. The same clustering strategy was employed on the Transcript Clusters associated with PVT lapses, again based on the average data from the 8 SD subjects. The fuzzification parameter was m=1.76, again with creation of three clusters.

Transcription Factor Regulators

We assigned Regulatory Impact Factor (RIF) scores to known human transcription factors as a means of ranking potential regulators of the differentially expressed genes. This approach involved a differential co-expression analysis, as described by Reverter *et al.* (2010). In synopsis, transcription factors were separately correlated to all differentially expressed genes in each of two conditions. Then the difference between the correlation of the transcription factor to gene expression in the first condition, and its

correlation to gene expression in the second condition, was computed and squared. This difference was next weighted by the average abundance of the differentially expressed gene across all samples, and its difference in expression between the two conditions. The final value was converted to a z-score and reported for each transcription factor to rank their regulatory potential. Based on the differences in correlation and in expression between the two conditions (here, C and SD), the RIF z-scores were either positive or negative. Larger absolute values of the z-scores were interpreted as stronger evidence for a regulatory role.

Three input sets of data were necessary for running this analysis: (1) a list of transcription factors, (2) a list of differentially expressed genes (Transcript Clusters), and (3) the expression values of the transcription factors and differentially expressed genes in each condition. The list of transcription factors (1) was created by inputting a list of gene symbols for all human Transcription Factors in QIAGEN BIOBASE TRANSFAC v. 2015.4 into the Affymetrix NetAffx™ tool (LIU *et al.* 2003) and exporting the associated list of Transcript Clusters. This list of transcription factor Transcript Clusters was reduced to those for which there was data after filtering for low expression levels (see above). For the second item two lists of differentially expressed genes were used running the RIF analysis twice. In one run the Treatment effect Transcript Clusters were used (2a), and in the other run the PVT effect Transcript Clusters were employed (2b) as the differentially expressed gene list. Finally, averages were computed for each of the 12 timepoints for C and for SD individuals, and these 12 values served as the input expression data (3) for each of the two conditions (C, SD). It was necessary computationally to have the same number of expression data points for both conditions, making it impossible to use the raw data for the 6 C vs. 8 SD subjects (fatigue resistant subjects were omitted). With these data in hand, the script published in the supplementary material of Uyhelji *et al.* (2016) was run to compute RIF z-scores, with minor modifications. Namely, Uyhelji *et al.* (2016) ran a Spearman correlation analysis on RNA-Seq discrete counts, whereas here Pearson correlations were applied to the log-transformed microarray data.

In addition to the RIF differential co-expression analysis, the BIOBASE F-match tool (http://www.biobase-international.com/) was utilized to search for regulatory transcription factors based on the promoter sequence of differentially expressed genes. Analysis was based on the BIOBASE TRANSFAC® v. 2015.4 vertebrate non-redundant profile (MATYS *et al.* 2006), with default settings modified to minSum for optimization of both false positive and false negative errors, and a P-value threshold of 0.05.

We scanned for over-represented binding sites in both Treatment effect and PVT Transcript Clusters. For the Treatment effect, a background set was randomly selected from Transcript Clusters with raw P-values >0.10 (FDR>0.337) in the limma differential expression analysis between C and SD subjects at the Experimental phase. For the PVT effect, the background set was randomly selected from Transcript Clusters with raw P-values >0.10 (FDR>0.513) for PVT lapses. Each background list contained the same number of Transcript Clusters as the foreground list (Treatment or PVT effect Transcript Clusters), per the manufacturer's recommendations. Ten background lists were created for each foreground set, and only transcription factors appearing on at least nine iterations of F-match were considered for further analysis.

Functional Enrichment and Pathway Analysis

Affymetrix's online tool NetAffx™ (LIU *et al.* 2003) was used to annotate gene lists, with emphasis on the first annotation provided for genes with mixed hybridization targets per Affymetrix's

recommendation (pers. comm.). Also, the DAVID v. 6.7 bioinformatics tool (HUANG *et al.* 2008) was used to characterize functional enrichment. For these, Transcript Clusters in the list of interest served as the foreground input, with all Transcript Clusters in the corresponding RMA expression set passing the low-expression filter as the background. As suggested by TIMMONS *et al.* (2015), such user-defined backgrounds can be important to reduce bias towards tissue-specific expression (e.g., detection of pathways found in blood due to using blood as the RNA source). Analysis was focused on DAVID functional clusters with enrichment scores >1.3, corresponding to $P < 0.05$.

The Ingenuity Pathway Analysis® (IPA®, QIAGEN Redwood City, http://www.qiagen.com/ingenuity) Core Analysis tool was used to explore molecular pathways and networks based on previously published interactions among genes. Parameters used included consideration of both direct and indirect relationships, exclusion of endogenous chemicals, inclusion of Causal Network analysis, use of Ingenuity expert and Ingenuity supported third party information from experimentally observed data (vs. predictions), and restriction of species to mammals (human, mouse, rat). The foreground in these IPA® runs consisted of the gene list of interest (e.g., Treatment or PVT effect Transcript Clusters, converted internally by IPA® to genes), and as with DAVID, the background was all microarray genes passing the $\log_2 > 6$ low-expression threshold in at least six samples. Preliminary runs using the default background of all genes represented in the HuGene 1.0 ST microarray suggested that these user-defined backgrounds typically were more conservative in yielding results.

Because PVT lapses were ordinal rather than binary, fold changes for the PVT effect could not be computed directly to portray the direction of expression. Instead, the $\log_2$ fold change values were used for SD relative to C subjects (omitting the 3 fatigue resistant persons) at the Experimental phase, taken from the Treatment effect analysis. Based on the sign of the correlation coefficient of a Pearson correlation between PVT lapses and gene expression, it was confirmed that the direction of expression indicated by the Treatment effect fold change reflected the direction relative to PVT. That is, Transcript Clusters with a positive Pearson correlation between PVT and gene expression (higher expression with more PVT lapses) also showed a positive fold change for the Treatment effect (higher expression in SD than C subjects). This information was used by IPA to depict whether a given gene was up-regulated or down-regulated, and whether connected molecules and biological functions were activated or inhibited. Causal Network pathways of interest were reviewed for connections between differentially expressed genes and upstream regulatory molecules (KRÄMER *et al.* 2014). Fisher's exact tests were used to check for the presence of a greater number of differentially expressed genes related to the Causal Network than would be expected due to chance.

MicroRNA Quantitative PCR

A 300 ng aliquot of total RNA from each of the 199 samples was used for the analysis of microRNA (miRNA) expression with 30 TaqMan® assays (Life Technologies, Grand Island NY) according to the recommendations from Fluidigm® for miRNA analysis on the BioMark system (MicroRNA Real-Time PCR Using Dynamic Array IFCs, PN 100-1616 B2 Fluidigm Corp., South San Francisco, CA). The Taqman microRNA Reverse Transcription kit (PN 1312165, Life Technologies) and KAPA Probe Fast qPCR Kit Master Mix Universal (PN KR0397-v5.13 KAPABiosystems, Wilmington, MA) were used in a modified protocol obtained from Fluidigm (PN 100-1616 B2 Fluidigm Corporation, Amsterdam) with custom Reverse Transcription and preamplification primer pool prepared as directed (PN 4465407 rev C, Life Technologies). Following a 17 cycle preampflication, the product was diluted 1:10 with 10mM Tris pH8.0, 0.1mM EDTA. The qPCR amplification reaction was prepared according to the manufacturer's

96x96 protocol (#68000130 Fluidigm) using KAPA Probe Fast qPCR Kit Master Mix Universal (PN KR0397-v5.13, KAPA Biosystems) on Biomark IFC Controller and HD instruments (Fluidigm® Corp.).

Initial analysis of the miRNA plate assays was done with settings as recommended (Fluidgm Real-Time PCR Analysis software, ver. 4.1.2), 0.65 quality threshold, Linear (Derivative) baseline correction and Auto (Global) Ct threshold method. Efficiencies for each gene were determined via duplicate standard curves run on the same plate. The miRNAs *MIRLET7A* and *MIRLET7D* were chosen as Normalizers using GeNorm and Normfinder. Finally, normalized gene expression values were analyzed via linear mixed-effects models with R package nlme v. 3.1-126, using marginal Type III sum of squares. Models predicted expression of each miRNA as a function of PVT lapses, Treatment (SD or C), and Time of Day (encoded as factor), including a random intercept for subject.

RESULTS

Generalized linear mixed-effects models of PVT scores for all 17 subjects with maximum likelihood tests revealed a significant effect of the SD Treatment on PVT lapses (**Fig. 2**, **Suppl. Table 2)**. Specifically, model selection by the lowest AIC and BIC scores preferred models including a Treatment by Phase interaction, where Phase distinguishes Baseline, Experimental, and Recovery periods (**Suppl. Table 2**).

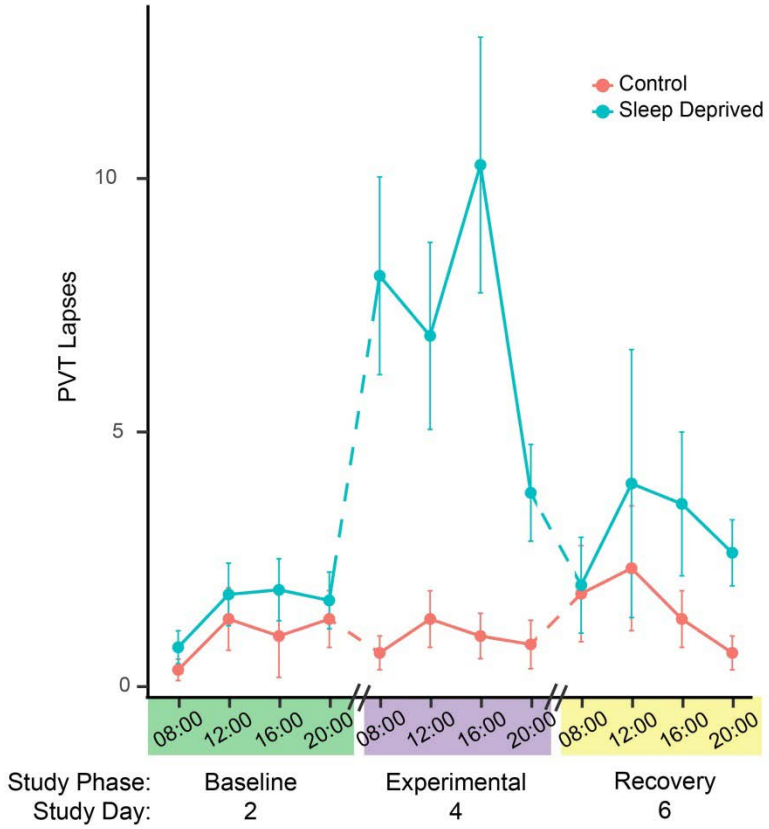

Figure 2.Mean (± 1 SE) PVT lapses of 11 SD and 6 C subjects during three of the seven consecutive study days, consisting of one day each during the Baseline, Experimental, and Recovery phase.

Although fatigue resistance in a larger sample size may be best represented by a continuum, here inspection of the PVT data suggested that three SD subjects were impacted less by total SD and they were categorized as fatigue resistant (**Suppl. Fig. 1**). It was hypothesized that this inter-individual variability could impede detection of a gene expression Treatment effect between C and SD persons, as the fatigue resistant subjects are outliers to the average SD response, at least in terms of PVT lapses. Thus, to test for genes differentially expressed respective to Treatment, the three fatigue subjects were omitted and comparison made between the 6 C and the remaining 8 SD persons. A brief comparison was made by running this model of a Treatment effect including the fatigue resistant individuals. About 90% of genes different between SD and C subjects at the Experimental phase in models with the fatigue resistant subjects also were significant in models without these subjects at FDR <0.05 (data not shown). However, models without these three subjects detected over three times as many differentially expressed Transcript Clusters. Some of these additional genes were known from literature review to respond to sleep levels such as *Interleukin 1B* (*IL1B*) (KRUEGER 2008; DA COSTA SOUZA and RIBEIRO 2015). Hence for biomarker discovery, models without the fatigue resistant subjects were considered more comprehensive and potentially more accurate indicators of a Treatment response. To discover biomarkers associated with PVT lapses, all 17 subjects were included because PVT lapses inherently provide a means of incorporating inter-individual variability into the statistical models.

Differential Gene Expression: Treatment and PVT effect

At the Treatment level, 225 Transcript Clusters, corresponding to 212 genes with annotated gene symbols or mRNA assignments (**Suppl. Table 3**), exhibited a significant difference between SD and C subjects in the Experimental but not Baseline phase. Most (~88%) were down-regulated in SD persons. Two Transcript Clusters representing *Cathelicidin Antimicrobial Peptide* (*CAMP*, FDR =3.56E-03, $\log_2$ fold change of -0.57) and *Defensin, Alpha 4, Corticostatin* (*DEFA4*, FDR =0.033, $\log_2$ fold change of -1.00) also were differentially expressed in the Recovery phase. No Transcript Clusters were significantly different in the Recovery phase but not the Experimental phase.

Thirty-four Transcript Clusters, representing 28 annotated genes (**Suppl. Table 4**), were associated with PVT lapses. There were 29 Transcript Clusters down-regulated and five up-regulated as PVT lapses increased and sleep was deprived. One of the up-regulated Transcript Clusters was *Outer Dense Fiber Of Sperm Tails 2-Like* (*ODF2L*), and the remaining four belonged to the *Speedy/RINGO* (*SPDY*) cell cycle regulator gene family. There were 15 Transcript Clusters corresponding to 13 distinct genes in the PVT list not found in the Treatment list (**Suppl. Table 4**), including *EF-Hand Domain Family, Member D2 (EFHD2); Ankyrin Domain Family (POTE); Growth Factor Receptor-Bound Protein 2 (GRB2); Potassium Inwardly-Rectifying Channel, Subfamily J, Member 15 (KCNJ15);* and *Flotillin 1 (FLOT1)*. To verify this result was not simply a reflection of the Treatment dataset excluding the fatigue resistant individuals while the PVT analysis included them, the Treatment analysis was run again with all 17 subjects (data not shown). During the Experimental phase, the lowest FDR for the difference between SD and C subjects in these 15 Transcript Clusters was 0.35, suggesting that the conclusion was robust to inclusion or exclusion of these subjects.

Co-expression and Temporal Networks

A total of 23 WGCNA modules of co-expressed Transcript Clusters were constructed, along with a group of three unassociated genes (Grey "module"). Each module was tested for an association with the key variables (Time of Day, Treatment, PVT). Some modules correlated with Time of Day, but analyses

focused on the association of modules with PVT. At a Bonferroni-corrected Type I error threshold of 0.0167, the White and Darkturquoise modules were significantly correlated with PVT lapses (**Fig. 3**, **Suppl. Table 5**), and Darkturquoise also was significantly correlated with Treatment. The genes represented in the Darkturquoise module primarily were involved in the immune response, with a large number of immunoglobulins including its top hub Transcript Cluster *Immunoglobulin Kappa Constant (IGKC)* (**Table 1**). In the White module, several Transcript Clusters represented members of the *SPDY* gene family, including the top hub Transcript Cluster (**Table 1**).

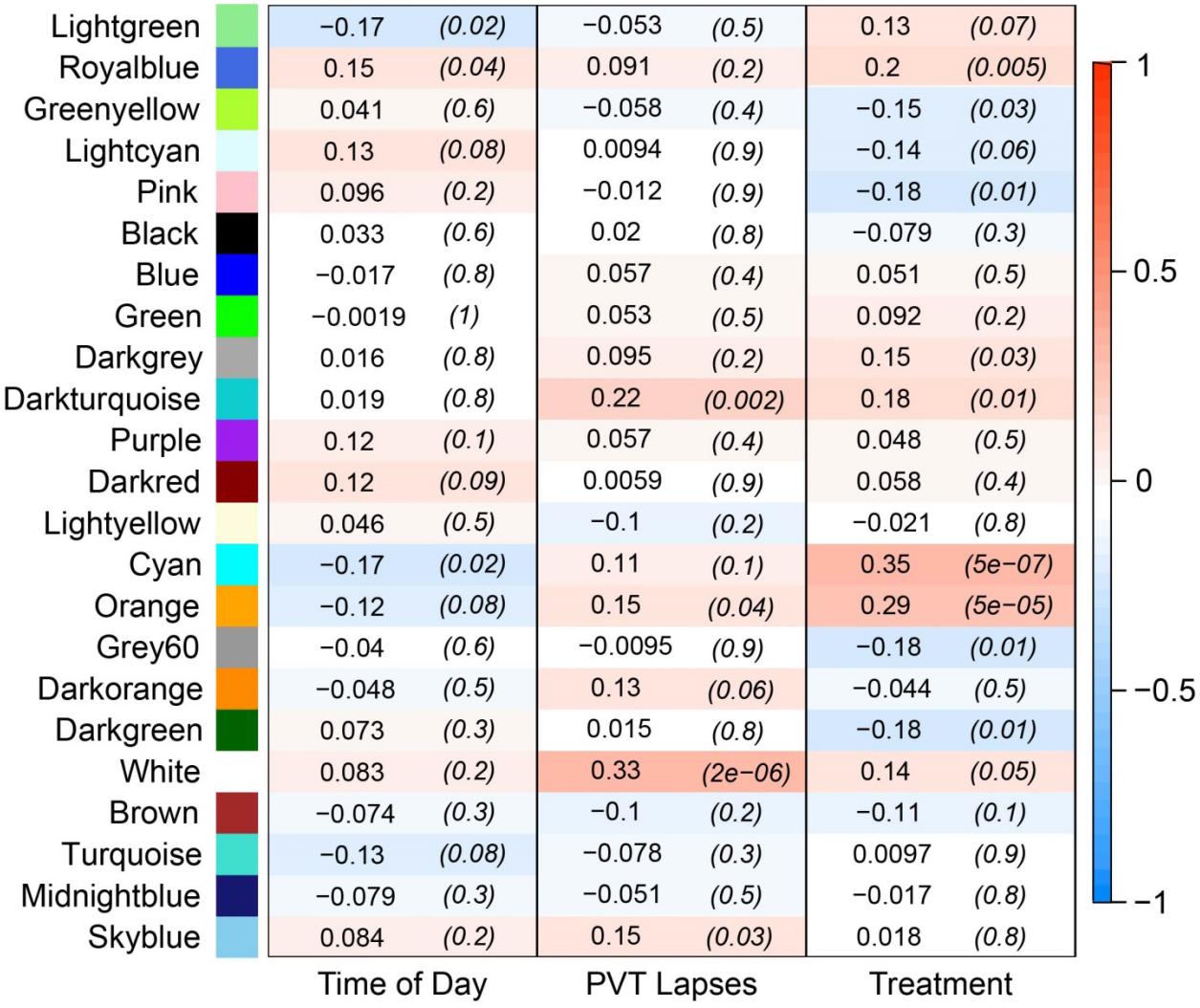

Figure 3. Matrix of Pearson correlations between WGCNA co-expression modules (rows), and each of three variables (columns): time of day, PVT lapses, and Treatment. Positive correlations are shown in red, and occur when gene expression was higher in samples at later times of day, in samples with higher PVT lapses, and in samples from SD relative to C subjects. Negative correlations are blue. Correlation coefficients are depicted for the correlation of each module and variable, with P-values in parentheses.

Table 1. Content of WGCNA modules, including the top hub gene in each module.

	PVT Transcript Clusters	Treatment Transcript Clusters	Total Transcript Clusters	Hub Transcript Cluster	Hub Gene Symbol
lightgreen	0	0	83	8031277	KIR2DS4
royalblue	0	8	69	7940287	MS4A1
greenyellow	0	5	980	7972069	MYCBP2
lightcyan	0	0	125	7994559	LOC101929910
pink	0	2	395	7987405	RASGRP1
black	1	3	1090	7974483	KTN1
blue	0	3	957	8127526	RPL39P5
green	0	4	540	8086148	RPL29P11
darkgrey	0	0	51	8019631	RNU2-1
darkturquoise	0	0	54	8043459	IGKC
purple	0	0	345	7995320	---
darkred	0	0	60	8133896	---
lightyellow	0	0	74	8089038	---
cyan	0	0	581	8030470	AP2A1
orange	0	0	49	8149927	CLU
grey60	0	3	125	8040080	RSAD2
darkorange	0	1	43	8019716	LOC101060376
darkgreen	0	1	58	7919412	NBPF19
white	4	6	39	8133600	SPDYE9P
brown	7	56	1060	7986010	IQGAP1
turquoise	21	127	1420	7960518	TNFRSF1A
midnightblue	1	5	160	7989037	CCPG1
skyblue	0	0	36	7896742	LOC101928706

Temporal clustering of the Treatment effect Transcript Clusters in SD subjects with Mfuzz resulted in a single group of 26 Transcript Clusters up-regulated during the Experimental phase (Mfuzz Treatment Group 2), whereas Transcript Clusters in the other two Mfuzz groups were down-regulated (**Fig. 4**, **Suppl. Table 3**). Down-regulated Mfuzz Treatment Group 1 contained 104 Transcript Clusters, and DAVID analysis revealed functional enrichment of ion binding and cell adhesion. Genes represented in Mfuzz Treatment Group 1 included *Argonaute RISC Catalytic Component 4 (AGO4), Prostaglandin-Endoperoxide Synthase 2 (PTGS2), Casein Kinase 1, Alpha 1-Like CSNK1A1L, Protein Kinase C, Beta (PRKCB), Lipopolysaccharide-Induced TNF Factor (LITAF), IL1B, Eukaryotic Translation Initiation Factor 4E Family Member 3 (EIF4E3),* and *Glycogen Synthase Kinase 3 Beta (GSK3B)*. The up-regulated Mfuzz Treatment Group 2 was smaller and lacked significantly enriched functional clusters in DAVID; members included Transcript Clusters associated with B cell signaling, and genes in the *SPDY* family. Down-regulated Mfuzz Treatment Group 3 contained 94 Transcript Clusters, with functional enrichment of immunoglobulins, cell motility, and the inflammatory response. Among the genes in this group were *Basic Helix-Loop-Helix Transcription Factor (HIF1A), Chemokine (C-X-C motif) Receptor 2 (CXCR2), Solute Carrier Family 11 (Proton-Coupled Divalent Metal Ion Transporter), Member 1 (SLC11A1), Chemokine (C-X-C motif) Receptor 1 (CXCR1), Interleukin 17 Receptor A (IL17A), Cytoplasmic Polyadenylation Element Binding Protein 4 (CPEB4),* and *Immediate Early Response 3 (IER3).*

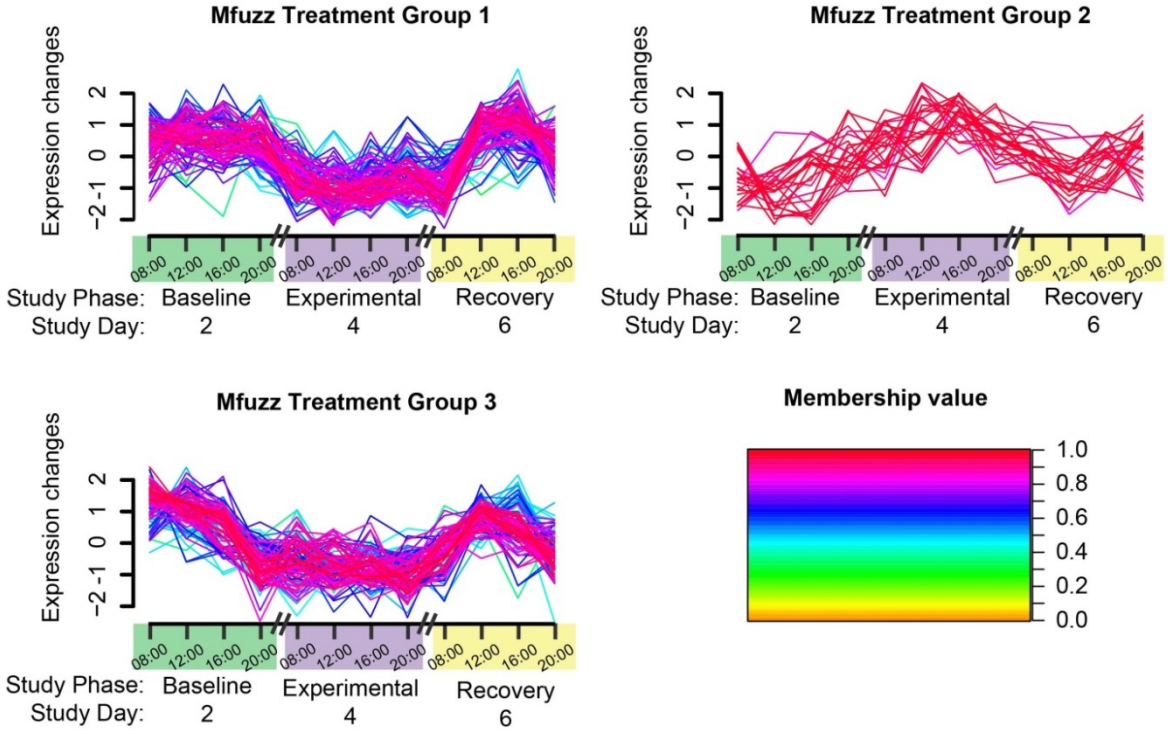

Figure 4. Temporal expression of microarray Transcript Clusters significant for the Treatment effect (lines, colored by strength of Mfuzz group membership). Values represent normalized expression in SD subjects during three of the seven consecutive study days, consisting of one day each during the Baseline, Experimental, and Recovery phase.

Although members of both Mfuzz Treatment Groups 1 and 3 exhibited down-regulation in SD persons during the Experimental phase, the temporal patterns differed slightly. Mfuzz Treatment Group 1 showed highest expression at midday in Recovery and a weak trend towards highest expression midday in Baseline, which was disrupted by down-regulation in the Experimental phase. Overall, Mfuzz Treatment Group 3 Transcript Clusters had their highest expression during Baseline at 08:00 h and decreased expression to 20:00 h. Not only was this pattern disrupted during the Experimental phase similar to Mfuzz Treatment Group 1, but also there was a delay in the maximum expression until 12:00 h in the Recovery phase.

When Transcript Clusters associated with PVT lapses were temporally clustered, there again were two Mfuzz down-regulated groups and one up-regulated group during the Experimental phase (**Fig. 5, Suppl. Table 4**). For down-regulated Mfuzz PVT Group 1 with 15 Transcript Clusters, cell motility was the only significantly enriched functional cluster found in DAVID. Members of Mfuzz PVT Group 1 included *Aquaporin 9 (AQP9*), chemokine receptors *CXCR1* and *CXCR2*, and *HIF1A*. In down-regulated Mfuzz PVT Group 2 with 14 Transcript Clusters, there was one significantly enriched functional group, namely ion transport. Included in Mfuzz PVT Cluster 2 were *LITAF*, *KCNJ15*, and *FLOT1*. There were no significant enrichment terms for Mfuzz PVT Group 3, which consisted of four Transcript Clusters for the *SPDY* family (**Fig. 5**, red and pink lines), plus *OD2FL*. Similar to Group 3 of the Mfuzz Treatment results, the Mfuzz PVT Group 1 showed a pattern of decreasing expression from 08:00 to 20:00 at Baseline that shifted to overall low expression in the Experimental phase, with continuing distortion

during the Recovery phase. The Mfuzz PVT Group 2 was roughly similar in expression pattern to Mfuzz Treatment Group 1 in having a circadian maximum midday in the Baseline and Recovery phases, which was depressed during the Experimental phase. Mfuzz PVT Group 3 exhibited a similar expression pattern to Treatment Group 2, with up-regulation during the Experimental phase.

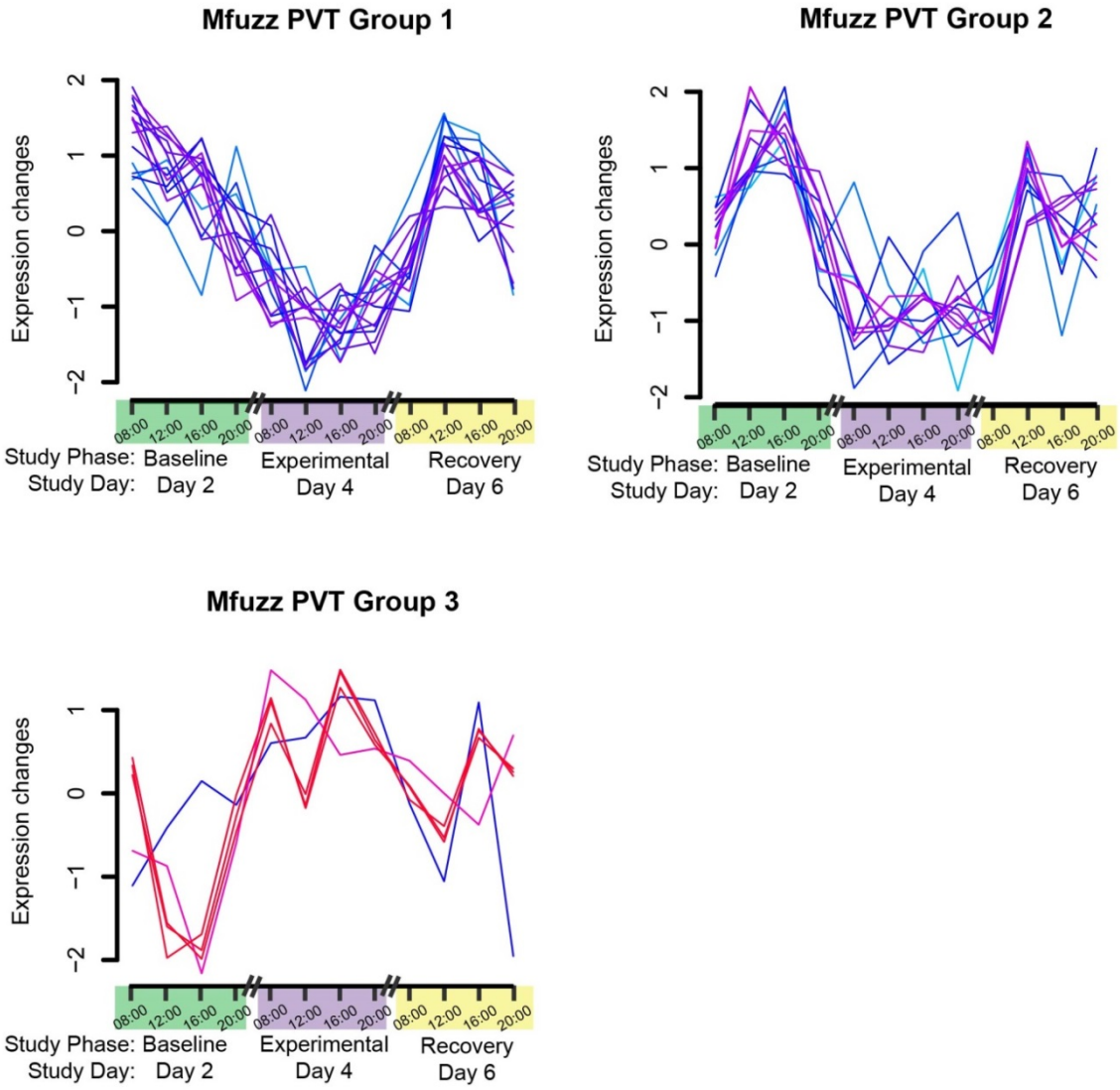

Figure 5. Temporal expression of microarray Transcript Clusters significant for the PVT effect (lines, colored by strength of Mfuzz group membership as in **Fig. 4**). Values represent normalized expression in SD subjects during three of the seven consecutive study days, consisting of one day each during the Baseline, Experimental, and Recovery phase.

Transcription Factor Regulators

RIF z-scores were used to rank human transcription factors as potential regulators for the Transcript Clusters associated with Treatment and PVT. For the Treatment list, no transcription factors had z-scores >2, whereas 41 had z-scores <-2 (**Suppl. Table 6**). The smallest RIF value corresponded to the *ELK3, ETS-domain protein - SRF accessory protein 2 (ELK3)*, with RIF z-score =-7.40. From the PVT list, 7 Transcript Clusters had z-scores >2 including the *basic helix-loop-helix family member 40* (*BHLHE40*, RIF z-score =2.64), and 25 had z-scores <-2 (**Suppl. Table 7**). The largest absolute value of the RIF score was for *ELK3*, with RIF z-score =-6.36.

To further characterize potential regulators of the response to sleep deprivation, the BIOBASE F-match algorithm was used to assess potential transcription factor binding sites in the promoters of Transcript Clusters from the Treatment and PVT lists. Seven transcription factor matrices were predicted to regulate genes that respond to Treatment: c-Myb, E2A, Ets, GKLF, E-box, GLI, and myogenin. Only Hic1 met the screening criteria for the PVT list.

Comparing the results from RIF with F-match, two matrices, Ets and E-box, were identified in F-match for the Treatment list, and associated with transcription factors having an RIF z-score >2 or <-2. The Ets and E-box binding matrices were found in many Treatment genes by F-match, and among transcription factors known to bind these matrices were four genes with high RIF z-scores: *Upstream Transcription Factor 1 (USF1)* with an E-box binding site; *Transcription Factor 4 (TCF4)* with an E-box binding site; *GA Binding Protein Transcription Factor, Alpha Subunit 60kDa (GABPA)* with an Ets binding site; and *ELK3* with an Ets binding site (**Table 2**). Three of these transcription factors, *ELK3, TCF4*, and *USF1*, had an RIF z-score <-2 in the PVT list; however, F-match PVT results only identified the Ets matrix as over-represented against three background sets, and the E-box motif against two backgrounds. Expression levels of the gene *Hypermethylated in Cancer 1 (HIC1)* did not meet our low-expression filter, and thus it was excluded from the RIF analysis.

Table 2. Transcript clusters with evidence of regulatory roles from RIF and Biobase F-match analysis. F-match over-representation was reported as the number of times the binding matrix was over-represented out of 10 runs.

Transcript Cluster	Treatment RIF z-score	PVT RIF z-score	Gene Symbol	Binding Matrix	Treatment F-match over-representation	PVT F-match over-representation
7957665	-7.40	-6.36	ELK3	Ets	10	3
8133030	-3.01	-0.82	GABPA	Ets	10	3
8023415	-2.07	-2.13	TCF4	E-box	9	2
7921738	-5.44	-3.35	USF1	E-box	9	2

Functional Enrichment and Pathway Analysis

Functional enrichment in DAVID was similar for both the Treatment and PVT effects, albeit with fewer findings for the much smaller PVT list (**Tables 3-4**). Several enriched functional categories were related to the immune response and motility, including immunoglobulins, components of the inflammatory response, cell adhesion, and calcium ion binding. There were no significantly enriched functional clusters for the up-regulated Transcript Clusters of the Treatment list (Mfuzz Treatment Group 2, **Fig. 4**) or PVT list (Mfuzz PVT Group 3, **Fig. 5**), likely due to the small total number of up-regulated

Transcript Clusters. Most Transcript Clusters were down-regulated. In support of the results from DAVID, the IPA® Core Analysis of the Treatment list predicted inhibition of immune cell trafficking, cell movement, inflammatory response, and cell-to-cell trafficking and signaling (**Suppl. Table 8**).

Table 3. Functional enrichment for DAVID clusters with scores >1.3 (corresponding to P<0.05) from analysis of the Treatment effect list.

Cluster Number	Enrichment Score	Number of genes	Description
1	4.43	99	membrane
2	2.82	14	immunoglobulin
3	2.74	16	cell adhesion
4	2.34	38	cell motility; inflammatory response
5	2.24	13	cell junction
6	2.03	12	coagulation
7	1.96	21	cell fraction
8	1.76	3	metal ion-binding site:Calcium
9	1.44	15	vesicle
10	1.43	3	sushi; complement control module
11	1.33	13	lipoprotein

Table 4. Functional enrichment for DAVID clusters with scores >1.3 (corresponding to P<0.05) from analysis of the PVT effect list.

Cluster Number	Enrichment Score	Number of genes	Description
1	1.65	9	cell motility; inflammatory response
2	1.37	9	vesicle-mediated transport; intracellular signaling cascade
3	1.37	19	membrane
4	1.35	5	calcium-binding EF-hand

The 28 genes associated with PVT lapses were part of multiple upstream Causal Networks in IPA®, 25 of which were connected to at least 10 of the differentially expressed PVT genes. One had similarity to a network from the Treatment list in that the *Bradykinin Receptor (BDKR)* was a master regulator in both (**Suppl. Fig. 2**, Network B). This network was significantly enriched for both the Treatment (P =8.75E-04) and PVT analysis (P =4.22E-03). Both Treatment and PVT Causal Networks provided evidence for purinergic activity, but with different purine type receptors as the master regulator: *P2RX4* for Treatment, (P =9.16E-04) and *P2RX7* for PVT (P =2.46E-03) (**Suppl. Fig. 3**, Network P). A PVT Causal Network of interest (P =1.72E-03) that contained evidence of ion channel activity had adenylate cyclase (ADCY) as its master regulator (**Fig 6**, Network A). Also, a PVT Causal Network pathway with the master regulator DNAJ (**Suppl. Fig. 4**, Network D) predicted up-regulation of several heat shock proteins including DNAJ, HSP70, and HSP90 (P =4.09E-03). However, there were no corresponding Treatment Causal Networks with ADCY or DNAJ as the master regulator.

MicroRNA Quanitative PCR

None of the tested miRNAs were significantly associated with Treatment. However, three were significantly related to PVT lapses (FDR<0.05): *microRNA 152 (MIR152), microRNA 27b (MIR27B),* and *microRNA 24 (MIR24)* (**Suppl. Table 9**).

DISCUSSION

This report represents one of the first studies of gene expression biomarkers for impaired neurobehavioral performance due to sleep deprivation. The lack of biomarkers for the consequences of sleep loss was highlighted at a recent workshop on sleep biomarker discovery (MULLINGTON *et al.* 2016). Some of the difficulty identifying biomarkers for sleep deprivation hitherto may have been caused by the large inter-individual variability in responses to sleep loss. In particular, the phenomenon of fatigue resistance has been recognized for over a decade (VAN DONGEN *et al.* 2004). To date no reliable method to identify resistant individuals prior to SD exposure has been developed. In the present study, inspection of PVT lapses (**Suppl. Fig. 1**) identified three out of 11 SD subjects as fatigue resistant. By testing for the relationship of gene expression in blood to PVT lapses that encompass some of this variability, additional biomarkers were found that were not identified by assessment of a simple Treatment effect. Of course the same was true in reverse, as we identified 212 Treatment effect genes in blood (**Suppl. Table 3**) and a mere 28 genes associated with PVT lapses (**Suppl. Table 4**). Detecting a relationship of gene expression with PVT lapses may be more difficult, considering the added complexity and perhaps narrower suite of associated genes for neurobehavioral traits.

While identifying genes associated with a sleep loss Treatment has value, biomarkers for functional impairment are more relevant to assessing fitness for duty in safety-critical roles and may aid fundamental understanding of the relationship between sleep and cognition. Shifting the focus from biomarkers for sleep loss over time to biomarkers for neurobehavioral impairment from SD strengthens characterization of the molecular basis of the phenotype, and also emphasizes operational needs. A key next step will be determining the neurobehavioral or other phenotypic metrics that best summarize fitness for duty; answers may vary depending on the tasks workers perform (VAN DONGEN *et al.* 2011b; MULLINGTON *et al.* 2016). Nonetheless, the capacity for sustained vigilance is needed across most if not all fields, making biomarkers linked to PVT lapses important indicators for use in both accident prevention and post-accident root cause analysis. Beyond the applied value of such research, it provides the fundamental value of directly assaying the molecular changes associated with neurobehavioral performance for improving fundamental understanding of the relation between sleep loss and capacity for sustained attention.

Most genes identified in this study exhibited down-regulation in SD relative to C persons at the Experimental phase, a pattern consistent with prior studies. Blood draws for subsequent nights are needed to determine how long the effect continues. As reviewed by Mackiewicz et al. (2009), sleep is associated with macromolecule biosynthesis, and prolonged wakefulness leads to down-regulation of genes associated with multiple metabolic processes. This study indicates potential effects on translation in the down-regulation of *CPEB4* (Treatment and PVT effect lists) and *EIF4E3* (Treatment list only). The *CPEB4* gene is one of four vertebrate cytoplasmic polyadenylation binding proteins that regulates translation via effects on poly(A) elongation (RICHTER 2007; CHARLESWORTH *et al.* 2013). The phosphorylated form of the CPEB protein promotes translation of mRNAs with roles in learning, memory, and synaptic plasticity (RICHTER 2007; GRØNLI *et al.* 2012), which may explain its relation to PVT lapses. Grønli and colleagues (2012) report that sleep deprivation leads to reduced phosphorylation

of Cpeb in the hippocampus and Eif4e in the dentate gyrus of rats. Although the *EIF4E* gene was not significantly related to PVT lapses in the present study, it was down-regulated in response to the SD Treatment. The protein EIF4E is a component of the translation initiation complex (GINGRAS *et al.* 2001); decreased levels of this protein would be detrimental to synthesis of new protein and could contribute to the known effects of sleep loss on macromolecular biosynthesis.

Sleep Deprivation Biomarkers and Immunity

Cytokine and stress-associated networks frequently are associated with sleep deficits (PELLEGRINO *et al.* 2012; DA COSTA SOUZA and RIBEIRO 2015), and results here support the association of SD with the immune system. IPA® Causal Networks detected in both Treatment and PVT analyses have *BDKR* as the master regulator (**Suppl. Fig. 2**, Network B). Bradykinin receptors are mediators of the inflammatory response (HALL 1997), as indicated by inclusion of differentially expressed genes such as the chemokine *CXCR1* and transcription factor *LITAF* in the PVT network. The *LITAF* gene is a key mediator of the inflammatory cytokine response to lipopolysaccharides (TANG *et al.* 2006). Multiple genes related to the immune system are down-regulated in both Treatment and PVT lists, including *LITAF*, *CXCR1*, and *CXCR2*. The genes *IL17RA* and *IL1B* were down-regulated for Treatment only. In contrast to our findings, several reviews suggest that sleep loss results in increased levels of cytokines such as *IL1* (KRUEGER 2008; MULLINGTON *et al.* 2010; CLINTON *et al.* 2011; DA COSTA SOUZA and RIBEIRO 2015). While many reports are based on protein assays, studies reviewed by Krueger (2008) have shown that in brain, *IL1* mRNA increases during sleep deprivation. However, results in the present study are based on blood rather than brain samples. Also much of the *IL1* data in the reviews are derived from studies of animals, particularly rodents, and results may differ in humans. Details of the experimental design such as the time of measurement also may influence results. For example, in human blood higher mRNA levels of *IL1B* are found in day vs. nighttime samples (MÖLLER-LEVET *et al.* 2013).

Nonetheless, findings regarding the relation of cytokines to sleep are varied. A study of 40 h sleep deprivation found both increases and decreases of inflammatory cytokines detected via immunoassay and ELISA kits (FREY *et al.* 2007), and another study of partial sleep deprivation found elevated Interleukin 6 (IL6) protein (SHEARER *et al.* 2001). Yet, a meta-analysis reported no association between experimental sleep deprivation and circulating levels of IL6, Tumor Necrosis Factor alpha, or C-Reactive Protein (IRWIN *et al.* 2016). In contrast, the review by Da Costa Souza and Ribeiro (2015) suggests sleep deprivation is associated with changes in all three. In the current study, findings include evidence of specific aspects of the immune system being up-regulated. For example, an up-regulated group of Treatment effect genes (MFuzz Treatment Cluster 2, **Fig. 4**) contains members associated with B cell signaling. This is consistent with the study by Aho and colleagues (2013) of leukocyte gene expression in humans following partial sleep restriction, in which B cell activation is among the top up-regulated Gene Ontology pathways. Besides cytokines, the immune genes *CAMP* and *DEFA4* are of interest. These molecules were significantly down-regulated in SD subjects in both Experimental and Recovery phases. Their continuing down-regulation suggest the need for more than one Recovery night sleep to restore molecular homeostasis, as do the Mfuzz plots showing potential circadian distortion in the Recovery phase (**Figs. 4–5**). Blood draws were not collected after the second Recovery night to determine whether the effect continued. Cytokine and inflammatory networks may eventually contribute to a larger biomarker panel for diagnosing SD, but by themselves may be too pleiotropic to discriminate between sleep loss and other phenotypes such as illness.

Besides immunity, sleep deprivation typically is associated with evidence of a stress response including induction of heat shock proteins (TERAO *et al.* 2003; MACKIEWICZ *et al.* 2007; MACKIEWICZ *et al.* 2009; DA COSTA SOUZA and RIBEIRO 2015). One of the PVT Causal Networks predicts up-regulation of stress response genes including *HSP70* and *HSP90* (Network D, **Suppl. Fig. 4**). Differentially expressed genes in this network included transcription factors *HIF1A* and *LITAF*. While *HIF1A* is known for its role in activating hypoxic response genes, recent work suggests that *HIF1A* induction from hypoxia caused by obstructive sleep apnea, may disrupt circadian rhythms (JASPERS *et al.* 2015). Other members of Network D include cytokine receptors *CXCR1* and *CXCR2*.

Homeostatic and Circadian Clock Genes

Overlap between the circadian and homeostatic sleep processes is increasingly documented in literature (DEBOER *et al.* 2003; RAY and REDDY 2016), and results here further suggest a link with neurobehavioral function. Among the high-scoring transcription factors in the RIF analysis was *BHLHE40* (also known as *DEC1*), which together with *BHLHE41* (*DEC2*) acts as a transcriptional repressor of the *CLOCK/BMAL1* promoter (HONMA *et al.* 2002; NAKASHIMA *et al.* 2008). Intriguingly, mutations of *BHLHE41* have been associated with fatigue resistance (PELLEGRINO *et al.* 2014). Additionally, three miRNAs were found to have expression profiles significantly related to PVT lapses: *MIR24, MIR27B,* and *MIR152* (**Suppl. Table 9**). MicroRNAs are known for their roles in regulating gene expression (FABIAN *et al.* 2010), and have been associated with sleep and neurodegenerative disease (KAY and DAVIS 2013). Due to their relation to PVT lapses, these three miRNAs were intriguing candidates for regulating the molecular mechanism linking sleep deprivation and sustained attention. In mice *Mir27b* regulates the clock gene *Bmal1* at the posttranscriptional level (ZHANG *et al.* 2016). Although not part of the adenylate cyclase Causal Network A generated with IPA® (**Fig. 6)**, bioinformatics analyses suggest that *Mir27b* interacts with the *Adenylate cyclase 6* (*Adcy6*) gene (FIGUEREDO *et al.* 2013). It has been proposed that *Mir24* plays a role in regulating the period genes in mice (CHEN *et al.* 2013), and based on sequence analysis in humans, *MIR24* is predicted to interact with *CRY2* and *PER2 (HEEGAARD et al. 2016)*. Finally we note microRNAs themselves can exhibit circadian rhythm in their expression. For example, *Mir152* exhibits diurnal oscillations in mice (SHENDE *et al.* 2011). Plasma samples in humans revealed diurnal oscillations in *MIR24* (HEEGAARD et al. 2016), although evidence is mixed for such rhythmicity in *MIR27B* (FIGUEREDO et al. 2013; HEEGAARD et al. 2016).

Transcription factor analyses highlight further regulators with possible roles in both homeostatic and circadian processes, such as *USF1*. In mammals, the CLOCK/BMAL1 protein heterodimer binds E-boxes in the promoters of the *PERIOD (PER1 and PER2)* and *CRYTOPCHROME (CRY1 and CRY2)* genes leading to their activation, and the protein products of these genes repress the CLOCK/BMAL1 complex and in turn their own expression, until degradation of *PER* and *CRY* products releases CLOCK/BMAL1 (SIEPKA *et al.* 2007; LANDGRAF *et al.* 2012; SHIMOMURA *et al.* 2013). Oscillations in this molecular clock contribute to initiating circadian rhythms. USF1, like the CLOCK/BMAL1 heterodimer, binds E-box regulatory sites with peak binding at night, antiphase to CLOCK/BMAL1 (SHIMOMURA *et al.* 2013). The authors proposed that USF1 may help generate circadian rhythms by maintaining an open chromatin state, enhancing the ability of CLOCK/BMAL1 binding to the E-boxes on the next circadian cycle (SHIMOMURA *et al.* 2013). In the current study, not only was there a strong prediction of a regulatory role for *USF1* in the RIF analysis, but also the Biobase F-match tool revealed over-representation of E-box binding sites in the differentially expressed genes.

Other genes with regulatory roles supported by both RIF and F-match analyses were *GABPA*, *TCF4*, and *ELK3*. The protein encoded by the *GABPA* gene is a transcription factor that may function in human cognition (REIFF *et al.* 2014). Previous research on chronic sleep restriction in humans suggested a possible association between *GABPA* and gene down-regulation (AHO *et al.* 2013), but more work is needed to elucidate the relation of *TCF4* and *ELK3* to sleep deprivation. It should be noted that RIF and F-match test for regulatory effects in distinctly different ways, one by correlating expression of transcription factors with expression of the genes of interest, and the other by directly scanning for binding motifs in promoters. Therefore the two methods should be considered complementary, not necessarily confirmatory.

Novel Biomarkers and Genes Specific to Neurobehavioral Impairment

Altogether 13 genes were associated with PVT lapses but not with Treatment (**Suppl. Table 4**), including *FLOT1*. In mice, flotillins are up-regulated with sleep and down-regulated with sleep deprivation (MACKIEWICZ *et al.* 2007), which in the present study would be seen as a Treatment effect. Due to their association with lipid rafts, flotillins may have a role in neurotransmitter signaling (MACKIEWICZ *et al.* 2007; MACKIEWICZ *et al.* 2009). In contrast to the results of Mackiewicz et al. (2007), lack of a Treatment effect in the current study could reflect a difference between mice and humans. Of course confirming the absence of a Treatment effect for the 13 genes specific to the PVT analysis will require additional studies with more individuals. Differential gene expression studies as reported here are correlative in nature, and a different approach such as gene silencing or knockout experiments are needed to demonstrate causation. Nevertheless, a tantalizing hypothesis is that these 13 genes are specifically related to the mechanism by which SD affects the capacity for sustained attention.

Another down-regulated gene that was specifically associated with PVT was *KCNJ15*, an inward rectifying potassium channel proposed to be a key component of the potassium circadian cycle (GUMZ and RABINOWITZ 2013). It has been suggested that cycling of sodium and potassium currents is an evolutionarily conserved mechanism of governing clock neurons in the brain (FLOURAKIS *et al.* 2015). Recent work points to the role of neuromodulators influencing extracellular ion concentrations in the brain, in turn impacting sleep/wake activity (DING *et al.* 2016). Further evidence linking PVT lapses and ion channels exists in the IPA® Causal Network A (**Fig. 6**). The direction of change of the differentially expressed genes within this network was consistent with inhibition of an L-type calcium channel complex and activation of *Potassium Calcium-Activated Channel Subfamily N Member 4* (*KCCN4*). In mice, knockouts of *KCCN4* lead to reduced sleep duration (TATSUKI *et al.* 2016).

Beyond suggesting a role of ion signaling in SD and the resulting neurobehavioral deficits, Network A (**Fig. 6**) was intriguing due to the implications for cyclic adenosine monophosphate (cAMP) signaling. In general, activation of adenylate cyclase leads to production of cAMP (SUNAHARA and TAUSSIG 2002), whereas here it was predicted that adenylate cyclase was down-regulated, which in turn would reduce cAMP levels. Other components of Network A include predicted down-regulation of complexes for protein kinase A (PKA) and phospho-cAMP response element binding protein (CREB), which are implicated in memory storage (GRAVES *et al.* 2001). A study in mice demonstrated that increasing cAMP in hippocampal neurons can rescue the typical memory consolidation impairment caused by sleep deprivation (HAVEKES *et al.* 2014). Via its impact on cAMP, this network also implicates the immune system. Narasimamurthy and colleagues (2012) proposed a model in which Cryptochrome 1 (CRY1) inhibits adenylate cyclase, reducing levels of cAMP and ultimately of IL6. In this study the *IL6* gene was excluded from analyses due to its low expression across multiple samples, but reduction of this cytokine

would be consistent with the predictions of inhibition of the immune system. Additional Causal Networks contain purine type 2X7 (PVT) and 2X4 (Treatment) receptors (**Suppl. Fig. 3**, Network P). Binding of ATP to P2X4 receptors is known to promote REM sleep, whereas binding to P2X7 receptors promotes non-REM sleep (CLINTON *et al.* 2011). As reviewed previously (VAN DONGEN *et al.* 2011a; HOLST and LANDOLT 2015), adenosine binding to P2X7 receptors has been implicated in effecting the cumulative deficits in PVT performance due to chronic sleep loss, but these receptors also can act independently of adenosine to promote the release of sleep regulatory substances. Adenosine itself is a sleep regulatory substance as stated in reviews (CLINTON *et al.* 2011; HOLST and LANDOLT 2015), although causal roles for the molecule in sleep homeostasis are controversial (HOLST and LANDOLT 2015).

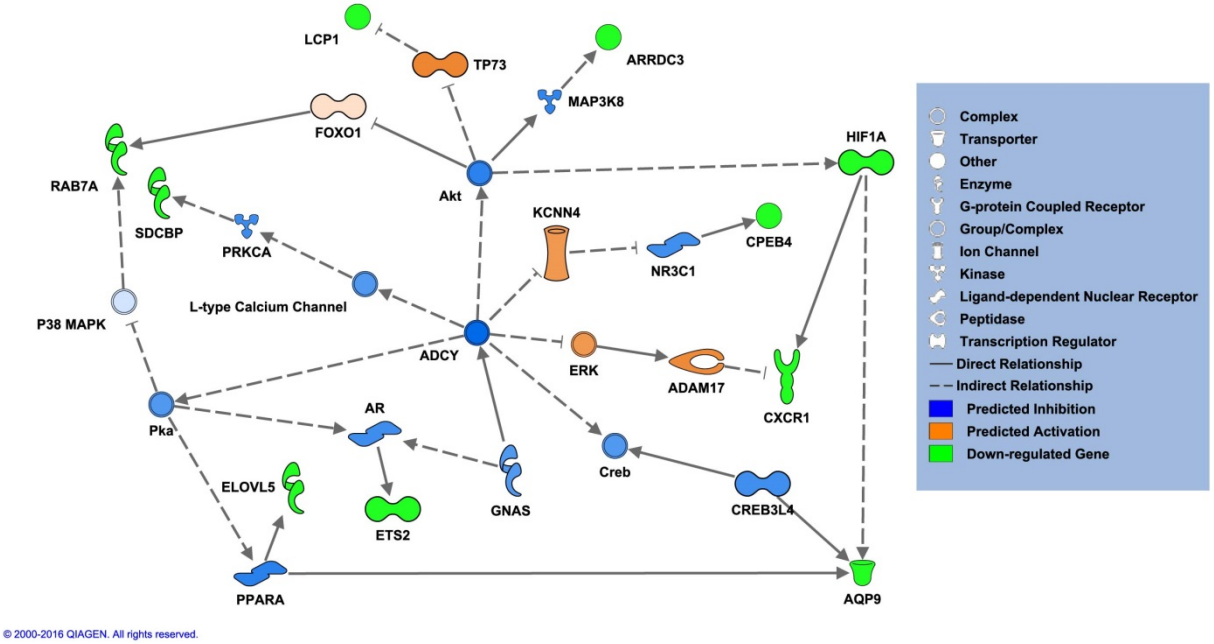

Figure 6. Ingenuity Pathway Analysis® Causal Network pathway A with master regulator adenylate cyclase (ADCY), predicted from the PVT effect gene list. Lighter molecule color suggests less confidence in prediction of the direction of expression (e.g., *P38 MAPK, FOXO1*); darker indicates more confidence.

Genes within the *SPDY* family constitute a new group of candidate biomarkers for the effects of SD. In differential expression analysis of both Treatment and of PVT lapses, Transcript Clusters for *SPDY* genes were up-regulated (**Figs. 4 and 5**), and WGCNA grouped several members of the *SPDY* family in a co-expression module (White) positively correlated with PVT lapses (**Fig. 3, Suppl. Table 5**). The *SPDY* members can activate cyclin-dependent kinases independent of cyclin activity, and they function in cell cycle progression, meiotic maturation, and the DNA damage response (GASTWIRT *et al.* 2007; CHAUHAN *et al.* 2012). The *SPDY* family has not previously been associated with sleep deprivation, although *Cyclin A* has been linked to sleep-wake transitions and the sleep homeostat in *Drosophila* (ROGULJA and YOUNG 2012). The best known member of this family, *SpdyA* (also known as *Spy1*), was shown to be expressed in the lumbar spinal cord of adult rats and may function in nerve regeneration (HUANG *et al.* 2009; CAO *et al.* 2013). Meanwhile, a growing body of research points to effects of sleep deprivation on adult neurogenesis, albeit results seem to vary among studies and may depend on the extent of sleep

deprivation (FERNANDES *et al.* 2015). Combining these findings one can hypothesize that sleep deprivation induces the *SPDY* family, thereby altering cell cycle progression and neurogenesis, potentially mediated by effector molecules downstream of ion-channels detailed above. This gene family has high sequence homology making unambiguous identification of the relevant family members difficult. Additional work is needed not only to confirm the link between *SPDY* expression and neurobehavioral impairment, but also to identify the specific *SPDY* genes responding to levels of sleep loss in blood cells.

Future Directions

Collecting nighttime blood samples from C individuals without disturbing their sleep would be helpful for refining the association of biomarkers with neurobehavioral impairment across the circadian cycle. Mounting evidence suggests overlap at the molecular level between the homeostatic and circadian rhythm sleep processes, complicating identification of biomarkers that can show impairment regardless of the time of measurement. Many of the genes associated with neurobehavioral impairment from SD in this study are connected to several different regulatory pathways, suggesting pleiotropic roles. For example, Networks B, A, D, and P for PVT all contain six of the 28 differentially expressed PVT genes, *AQP9, ARRDC3, CPEB4, ELOVL5, HIF1A,* and *LCP1* (**Fig. 6, Suppl. Fig. 2, Suppl. Fig. 3, Suppl. Fig. 4)**. Moreover, these four networks all contain two ligand-dependent transcription factors, *Nuclear Receptor Subfamily 3, Group C, Member 1 (NR3C1),* and *Peroxisome Proliferator Activated Receptor Alpha (PPARA)*. While all four networks clearly predict down-regulation of *PPARA*, for the glucocorticoid receptor *NR3C1* evidence of down-regulation is stronger in networks A and P (**Fig. 6, Suppl. Fig. 3**). The PPARA protein is important to coordinating rhythmic gene expression, and is a receptor for the period gene PER2 (SCHMUTZ *et al.* 2010). Ultimately, confirmation of predicted pathway networks and regulatory molecules will require targeted laboratory studies (e.g., gene knockout or silencing assays). The present investigation was aimed at biomarker discovery and, as with any such project, will require further work for validation (MULLINGTON *et al.* 2016).

In this study (**Suppl. Table 3, Suppl. Table 4**), as in prior research on sleep and gene expression (ARNARDOTTIR *et al.* 2014), fold change values were typically low. Translation of biomarker panels from research to the operational setting will require additional data collection from more subjects to increase power, and to adequately represent the continuum of SD responses. However, identification of similar themes in multiple different analyses increases confidence in the results presented here. For example, the WGCNA co-expression module most strongly correlated with PVT lapses contains several *SPDY* genes, congruent with identification of *SPDY* members in the PVT differential expression list.

It is anticipated that the association of gene expression with neurobehavioral impairment from sleep loss will be of great fundamental and applied interest. The 13 genes unique to the PVT analysis may be particularly promising candidates for exploring the relationship between SD and the capacity for sustained attention at a molecular level. This is particularly relevant in light of findings that SD affects multiple distinct aspects of cognition differentially (TUCKER *et al.* 2010; JACKSON *et al.* 2013; WHITNEY *et al.* 2015). Although the PVT is perhaps the most widely used neurobehavioral assay in sleep deprivation research, tests are available for other components of cognition, and gene expression patterns associated with aspects of decision making should be examined. Such comparisons may yield new biomarker panels or identify genes that overlap with the PVT list here, providing new insights regarding the molecular changes associated with the response of diverse aspects of neurobehavioral performance to sleep loss. Results from other fields such as proteomics and metabolomics may yield further biomarker candidates and capture additional phenomena, such as post-translational effects. Separate analyses of

fatigue resistant individuals to determine any gene expression patterns unique to them could advance understanding of the ability to maintain neurobehavioral functioning during SD. Because the current study only included three fatigue resistant subjects, this is left to future studies with a larger sample size.

AUTHOR CONTRIBUTIONS

Project design and oversight were done by Dennis Burian, Melinda Jackson, and Hans Van Dongen. Laboratory sleep deprivation and neurobehavioral assessment infrastructure, methodology, staffing, and expertise were provided by Hans Van Dongen. Subject recruitment, study logistics, and neurobehavioral assessment were overseen by Melinda Jackson. Vicky White performed sample preparation and assessment. Doris Kupfer performed microarray and qPCR runs and some preliminary analyses. Hilary Uyhelji performed all final analyses and developed approaches for relating gene expression to PVT. Hilary Uyhelji and Doris Kupfer wrote this manuscript, and all co-authors agreed to its content.

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LIST OF SUPPLEMENTARY MATERIAL

Suppl. Fig. 1. PVT lapses for C vs. susceptible and fatigue resistant SD subjects. One chart (top) compares the 6 C individuals to the 8 SD subjects susceptible to the SD treatment, whereas the other (bottom) compares the 6 C individuals to the 3 SD subjects identified as fatigue resistant.

Suppl. Fig. 2. Ingenuity Pathway Analysis® Causal Network pathway B with master regulator *BDKR* for PVT effect genes (top), and Treatment effect genes (bottom). Lighter molecule color (e.g., *NR3C1*, *ADAM17*) suggests less confidence in prediction of the direction of expression; darker indicates more confidence. In the PVT network, no prediction was made for *APP*, which was tested for differential expression but not significant. In the Treatment network, PKC complex prediction of light green reflects down-regulation of complex member *PRKCB*, but no differential expression for complex genes *PRKCA, PRKCD,* or *PRKCQ*.

Suppl. Fig. 3. **.** Ingenuity Pathway Analysis® Causal Network pathway P with purinergic receptor master regulator for PVT (top), and Treatment (bottom). Lighter molecule color suggests less confidence in

prediction of the direction of expression; darker indicates more confidence. In the PVT network, the software was unable to make predictions of direction of expression for *P2rx7, ERK1/2, PTK2B,* or *PRKCD*. Of these, *PTK2B* and *PRKCD* passed the low-expression thresholds but were not significantly related to PVT lapses.

Suppl. Fig. 4. Ingenuity Pathway Analysis® Causal Network pathway D for PVT effect genes with master regulator *DNAJ*. Lighter molecule color (e.g., *NR3C1*) suggests less confidence in prediction of the direction of expression; darker indicates stronger prediction.

Suppl. Table 1. Description of the samples. Values of N/A for the RNA integrity number correspond to three samples where this data could not be calculated. No RNA was collected for five timepoints with unsuccessful blood draws.

Suppl. Table 2. Sequential chi-square tests of models of PVT lapses, along with AIC and BIC comparisons for adding terms and their interactions. All models included a random intercept for subject and observation.

Suppl. Table 3. List of Treatment effect Transcript Clusters with fold change, P-value, and FDR for the Treatment effect at the experimental phase, Mfuzz Treatment group, and annotations from the NetAffx batch annotation tool. Notes on gene family, comments, and mRNA assignment are from review of the Affymetrix annotation package (HuGene-1_0-st-v1.na35.hg19.probeset.csv) for Transcript Cluster IDs without gene level (e.g., title) annotation or with mixed hybridization targets. Mixed hybridization targets suggest the potential for the Transcript Cluster to be associated with more than one gene. Three slashes designate multiple annotations associated with the same Transcript Cluster.

Suppl. Table 4. List of PVT effect Transcript Clusters with P-value and FDR for the PVT effect, Mfuzz PVT group, and annotations from the NetAffx batch annotation tool. Notes on gene family, comments, and mRNA assignment are from review of Affymetrix annotation package (HuGene-1_0-st-v1.na35.hg19.probeset.csv) for Transcript Cluster IDs without gene level (e.g., title) annotation or with mixed hybridization targets. Mixed hybridization targets suggest the potential for the Transcript Cluster to be associated with more than one gene. Three slashes designate multiple annotations associated with the same Transcript Cluster. Genes also found in the Treatment list are noted.

Suppl. Table 5. WGCNA module membership for all Transcript Clusters passing the low-expression threshold. Correlation coefficients (r) and P-values are given for the Pearson correlation between each Transcript Cluster and each of three variables: Treatment (SD vs. C), PVT lapses, and Time of Day. Annotations are taken from the Affymetrix batch NetAffx tool, with /// separating multiple annotations given to the same Transcript Cluster.

Suppl. Table 6. RIF z-scores for differential co-expression of human transcription factors with the Treatment effect Transcript Clusters. Annotations are taken from the Affymetrix batch NetAffx tool, with /// separating multiple annotations given to the same transcription factor Transcript Cluster.

Suppl. Table 7. RIF z-scores for differential co-expression of human transcription factors with the PVT effect Transcript Clusters. Annotations are taken from the Affymetrix batch NetAffx tool, with /// separating multiple annotations given to the same Transcript Cluster.

Suppl. Table 8. Functional categories and their prediction of activation or inhibition in Ingenuity Pathway Analysis® based on the Treatment effect list.

Suppl. Table 9. Results of qPCR on select miRNAs, including tests for a relation to PVT, Treatment, and Time of Day.

Requests for supplementary material mentioned in this report may be addressed to Dr. Hilary A. Uyhelji, Supervisory Research Geneticist, Biomedical Research Section (AAM-612), Civil Aerospace Medical Institute, P.O. Box 25082, Oklahoma City, OK 73125. Phone: (405) 954-7512 or the Aeromedical Research Division (405) 954-4808. Email: hilary.uyhelji@faa.gov