RAI1 Overexpression Promotes Altered Circadian Gene Expression and Dyssomnia in Potocki–Lupski Syndrome

Sureni V. Mullegama^{1,2,*} Joseph T. Alaimo^{1,*} Michael D. Fountain^{1,*} Brooke Burns³
Amanda Hebert Balog³ Li Chen^{1,4} Sarah H. Elsea^{1,2,3}

¹Department of Molecular and Human Genetics, Baylor College of Medicine, Houston, Texas, United States

²Department of Pathology and Laboratory Medicine, University of California, Los Angeles, California, United States

³Department of Human and Molecular Genetics, Virginia Commonwealth University School of Medicine, Richmond, Virginia, United States

⁴Department of Cellular and Genetic Medicine, School of Basic Medical Sciences, Fudan University, Shanghai, China

Address for correspondence Sarah H. Elsea, PhD, FACMG, Department of Molecular and Human Genetics, Baylor College of Medicine, One Baylor Plaza, NAB2015, Houston, TX 77030, United States (e-mail: elsea@bcm.edu).

J Pediatr Genet 2017;6:155–164.

Abstract

Retinoic acid induced 1 (RAI1) encodes a dosage-sensitive gene that when haploinsufficient results in Smith–Magenis syndrome (SMS) and when overexpressed results in Potocki–Lupski syndrome (PTLS). Phenotypic and molecular evidence illustrates that haploinsufficiency of *RAI1* disrupts circadian rhythm through the dysregulation of the master circadian regulator, *circadian locomotor output cycles kaput (CLOCK)*, and other core circadian components, contributing to prominent sleep disturbances in SMS. However, the phenotypic and molecular characterization of sleep features in PTLS has not been elucidated. Using the Pittsburgh Sleep Quality Index (PSQI), caregivers of 15 school-aged children with PTLS reported difficulties in initiating sleep. Indeed, more than 70% of individuals manifested moderate to severe sleep latency, as defined by the PSQI. Moreover, these individuals manifested difficulties in sleep maintenance, with middle of the night and early morning awakenings. When assessing daytime sleepiness through the Epworth Sleepiness Scale, approximately 21% of the individuals manifested excessive daytime somnolence. This indicates that mild dyssomnia characterizes the majority of the sleep phenotype, with occasionally problematic daytime somnolence, a phenotype different than that expressed by individuals with SMS, where daytime sleepiness is a chronic problem. Gene expression analysis of the core circadian machinery in the hypothalamus of the PTLS mouse model (*Rai1-Tg*) found significant dysregulation of the transcriptional activators, *Clock* and *Arntl*, and the transcriptional repressors, *Per1–3* and *Cry1/2*, during both light and dark phases. These findings suggest a partial loss of circadian entrainment typically evoked by environmental photic cues. Examination of circadian clock gene expression in the *Rai1-Tg* mouse heart, liver, and kidney found unchanged expression of *Clock* and most of its downstream

Keywords

- ▶ Potocki–Lupski syndrome
- ▶ RAI1
- ▶ circadian rhythm
- ▶ sleep disturbance

* Authors contributed equally to this work.

received
November 22, 2016
accepted after revision
January 17, 2017
published online
March 7, 2017

Copyright © 2017 by Georg Thieme
Verlag KG, Stuttgart · New York

DOI <https://doi.org/10.1055/s-0037-1599147>.
ISSN 2146-4596.

targets during both light and dark phases, suggesting an asynchronized circadian rhythm. Furthermore, examination of circadian gene expression in synchronized PTLs lymphoblasts revealed reduced transcripts of the Period (*PER1–3*) family and normal expression of *CRY1/2*. The finding that central circadian gene expression was altered while many peripheral circadian components were intact suggests a tissue-specific circadian uncoupling of the circadian machinery due to *Rai1* overexpression. Overall, our results demonstrate that overexpression of *RAI1* results in sleep deficiencies in individuals with PTLs due to a lack of properly regulated circadian machinery gene expression and highlight the importance of evaluating sleep concerns in individuals with PTLs.

Introduction

Chromosome band 17p11.2 is an error prone region of the genome that frequently undergoes recombination due to an enrichment of highly homologous low-copy repeats.^{1,2} Duplications within this region are associated with Potocki–Lupski syndrome (PTLS, OMIM 610883), whereas deletions are associated with Smith–Magenis syndrome (SMS, OMIM 182290).^{1–4} Within 17p11.2 resides the dosage-sensitive gene *retinoic acid induced 1* (*RAI1*, MIM 607642), and multiple studies have shown that a deletion or mutation in *RAI1* results in SMS, whereas duplication of *RAI1* results in PTLs.⁴ The full clinical phenotype of each syndrome is distinct and variable.^{4–7} The majority of individuals with PTLs present with intellectual disability, failure to thrive, minor dysmorphic features, cardiovascular anomalies, moderate-to-severe behavioral difficulties including anxiety and inattention, and autism spectrum disorder (ASD).^{8–15} The *Rai1*-transgenic mouse (*Rai1*-Tg) exhibits similar phenotypes as those observed in patients with PTLs, specifically increased anxiety and hyperactivity, growth retardation, and altered motor and sensory coordination.^{6,16}

Sleep disturbance is a prominent morbidity for individuals with SMS. Yet, the sleep phenotypes of its reciprocal contiguous gene syndrome, PTLs, have not been extensively characterized. Previously, a single study found that patients with PTLs suffer from multiple nocturnal awakenings and obstructive sleep apnea, describing the presence of sleep dysfunction.¹¹ When assessing the in vivo effects of *Rai1* overexpression, *Rai1*-Tg mice circadian periods are shortened by approximately 6 minutes.⁶ Recent molecular studies have found that *RAI1* functions as an enhancer of a main circadian regulator, *circadian locomotor output cycles kaput* (*CLOCK*, MIM 601851), and other key circadian genes, including the *PER* and *CRY* genes.^{17,18} Since *RAI1* is a dosage-sensitive gene, it is plausible that overexpression of *RAI1* could convey a circadian, anthropometric, and metabolic phenotype in individuals with PTLs.

In this study, we took a cross-translational approach to analyzing sleep in both humans and mice with PTLs. First, we employed a series of sleep surveys to identify specific abnormalities in a cohort of patients with PTLs. Next, we molecularly characterized the gene expression pattern of the core circadian machinery in PTLs patient cell lines and *Rai1*-Tg mice tissues, including the hypothalamus and several peripheral tissues. Our results indicate that individuals

with PTLs exhibit an intrinsic sleep dyssomnia. Moreover, altered circadian gene expression was observed in the hypothalamus and in some, but not all, peripheral tissues in *Rai1*-Tg mice, suggesting tissue-specific circadian uncoupling due to *Rai1* overexpression. Overall, our data demonstrate that overexpression of *RAI1* results in sleep deficiencies and a lack of properly regulated circadian machinery gene expression.

Materials and Methods

Participants and Survey Procedures

All participants were recruited through the PTLs outreach group through Facebook and e-mail solicitation of families enrolled in our PTLs research projects and reported anonymously. The surveys were administered online through www.surveymonkey.com for 7 months. Informed consent was obtained from parents or legal guardians of all participants. All aspects of the study were approved by the Virginia Commonwealth University Institutional Review Board. Of the 14 respondents, 78.6% had a maternal relationship with the child, 14.3% were fathers of the child with PTLs, and one individual responded as an adoptive grandparent. Participants were highly educated (100% of respondents having at least some college education) and typically worked full- or part-time (50% of the respondents worked full time, 28.6% worked part-time, 14.3% did not work outside the home, and one respondent was retired). Further demographic data are presented in **Supplementary Table 1**, available in the online version. All individuals with PTLs had a molecularly confirmed diagnosis either by array comparative genomic hybridization (50%), fluorescence in situ hybridization (42.9%), or karyotype (7.1%). The PTLs cohort was predominately male (64.3%), and 85.7% of the cohort slept in the same house as the parent every night (14.3% staying 4–6 nights per week with respondent). The average age of children with PTLs at the time of study was 6.68 (± 2.5) years. Ages of children ranged between 2 and 12 years, with 42.9% under 6 years of age and 57.1% between 6 and 12 years of age.

Measures

Pittsburgh Sleep Quality Index

The Pittsburgh Sleep Quality Index (PSQI) is a self-reported measure of sleep quality and degree of sleep disturbance.^{19–21}

The survey consists of 19 items that generate a composite global score. There are seven subscores that inform of the subject's sleep quality, sleep latency, sleep duration, habitual sleep efficiency, sleep disturbances, use of sleeping medication, and daytime dysfunction. The PSQI questionnaire was modified to allow for parents/guardians to answer the questions for their child on a 4-point Likert-type scale (0 = not at all; 1 = less than once per week; 2 = once or twice per week; 3 = three or more times per week). Children with total PSQI scores of ≥ 5 were identified as having severe sleep problems.²⁰

Epworth Sleepiness Scale

The Epworth Sleepiness Scale (ESS) is a measure to assess excessive daytime sleepiness.²² It is an eight-item self-report in which respondents indicate, on a 4-point Likert-type scale (0 = "would never"; 1 = "slight chance"; 2 = "moderate chance"; 3 = "high chance"), the likelihood that they will "doze off or fall asleep." Parents of 14 individuals provided surrogate answers for their children based upon observations and experience. A total score from 0 to 24 is possible. Higher scores indicate greater sleepiness during daily activities.²³ A total score >10 indicates excessive daytime sleepiness.²²

Cell Culture

Lymphoblastoid cell lines (LCLs) of patients (SMS224 and GM23053) and controls (GM23054 and GM23055) were used. Patient sample SMS224 was originally referred for clinical molecular evaluation under the approval of the Institutional Review Boards of Virginia Commonwealth University. LCLs from SMS224 were established as described previously.²⁴ GM23053, GM23054, and GM23055 were obtained from Coriell Cell Repositories (Camden, New Jersey, United States) and cultured as previously described.¹⁸ Ribonucleic acid (RNA) was isolated from LCLs through TRIzol (Invitrogen, Carlsbad, California, United States) according to standard protocols. All cell lines were cultured for the same period of time to the same cell density, and all samples were processed in the same manner.

Quantitative Real-Time Polymerase Chain Reaction

RNA was isolated from cultured patient and control LCLs through TRIzol according to standard protocols. Mice euthanized in the daytime hours (light phase) were taken at approximately 8 hours Zeitgeber time, and mice euthanized in the nighttime hours (dark phase) were taken at approximately 16 hours Zeitgeber time. Dissected mouse tissues included hypothalamus, heart, liver, and kidney, and RNA was isolated using the RNeasy Mini Kit (Qiagen, Venlo, Limburg, Germany). Quantitative reverse transcription real-time polymerase chain reaction (qRT-PCR) was performed as previously described¹⁸ through Taqman probes (Applied Biosystems, Foster City, California, United States) for *Gapdh* (MIM 138400; Hs9999905_m1; internal control), *Rai1* (Hs01554690_m1), and a subset of circadian rhythm and obesity genes (–Supplementary Table 2, available in the online version). All samples were tested and analyzed according to previously published methods using Bio-Rad CFX Connect Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, California, United States).

Overexpression of *RAI1* in HEK293T

HEK293T cells were transfected with *RAI1*^{Flag} plasmid as previously described.²⁵ Briefly, HEK293T cells were seeded at 5×10^6 cells/mL density in a six-well dish containing Dulbecco's modified Eagle's medium with 10% (v/v) fetal bovine serum, 2 mM L-glutamine, and 100 μ g/mL of penicillin/streptomycin. Control cells received Lipofectamine 2000 (Invitrogen) and pGL3 plasmid deoxyribonucleic acid (DNA), whereas experimental cells received Lipofectamine 2000 and *RAI1*^{Flag} for 24 hours according to manufacturer's instructions. Posttransfection RNA isolation and qRT-PCR analysis was performed as stated previously.

Rai1 Transgenic (*Rai1*-Tg) Mice Breeding and Maintenance

Mice used in this study have been previously published, and standard previously reported methods were followed for mice breeding and maintenance.^{5,6,17,25} Briefly, congenic C57Bl/6 *Rai1*-Tg mice were bred with wildtype (WT) C57Bl/6J mice to obtain animals for this study.^{5,6,17,25} Due to previously reported poor maternal behavior of the transgenic females, nontransgenic (NT) or C57Bl/6J females were added to the cage 2 to 3 days prior to birth to improve pup survival.¹⁶ Pups were weaned, tail clipped, tagged, and separated by sex at 3 weeks of age. A constant temperature (21°C), humidity (40%), automatic 12-hour light/dark cycle, and food ad libitum were maintained at all times unless otherwise specified. Male ($N = 6$) and female ($N = 6$) around 30-week-old WT and *Rai1*-Tg mice euthanized in the daytime hours (light phase) were taken at approximately 8 hours Zeitgeber time, and mice euthanized in the nighttime hours (dark phase) were taken at approximately 16 hours Zeitgeber time. All animals were housed in compliance with the guidelines of the Institutional Animal Care and Use Committee (IACUC) at Virginia Commonwealth University. All procedures were approved by the IACUC at Virginia Commonwealth University.

Rai1-Tg Genotyping

DNA was extracted from mice tails and genotyped as previously described.^{26–28} Briefly, the genotypes of the mice were confirmed using PCR analysis with unique bacterial artificial chromosome-end specific primers, as reported by Girirajan et al.^{5,6,17,25}

Statistical Analysis

Surveys

All data were analyzed in PRISM 4 version 4.0b (GraphPad Software, San Diego, California, United States). All statistical comparisons were performed using unpaired two-tailed *t*-tests at a 5% significance level. Due to the small sample size, descriptive statistics were used for the data analysis.

All Other Data

All data were analyzed in PRISM 4 version 4.0b. All statistical comparisons were performed using analysis of variance with posthoc testing where appropriate.

Results

Assessment of the Nature and Frequency of Sleep Impairments in Patients with PTLS

Parents of children with PTLS were recruited to this study to assess sleep deficiencies in their children. The two most frequent sleep problems reported by parents in response to the PSQI questionnaire were (1) the inability of their child to fall asleep within 30 minutes more than once or twice per week (78.6%) and (2) waking up in the middle of the night and early morning more than once or twice per week (78.6%) (►Table 1). Overall, parents reported that among their children, 38.5% had “fairly bad” quality of sleep and 38.5% had “fairly good” quality of sleep, whereas 23.1% described “very good” sleep quality (►Table 2). Sleep latency (PSQI component 2) was evaluated by scoring the time it takes to fall asleep and the number of times in a week an individual takes longer than 30 minutes to fall asleep. In the PTLS population studied, 71.4% of parents reported their children to have moderate or severe sleep latency (►Table 2), highlighting a high frequency of dysfunction in falling asleep. When assessing difficulty to stay awake during everyday activities and enthusiasm to complete daily tasks, to score daytime

dysfunction, 78.6% reported their children to have mild-to-moderate dysfunction.

Since the majority of the PTLS cohort experienced nocturnal awakenings, other sleep disturbances, and daytime dysfunction, we assessed nighttime behavioral problems. Interestingly, the majority of these children did not have commonly reported nighttime behavioral problems such as wandering around in the night, seeking food at night, or sleep-walking (►Table 3). Of note, many parents reported the use of various methods to promote proper sleep for their children, such as the use of sleep containment (35.7%) and medications for sleep aid (42.9%) (commonly melatonin; one case of clonidine), complicating the assessment of these behaviors (►Tables 2 and 3, and ►Supplementary Table 3, available in the online version).

Because chronic sleep problems can impact daily function, we assessed daytime somnolence using the ESS (see the Materials and Methods section).²⁹ ESS scores ≥ 10 are indicative of excessive daytime somnolence. Surprisingly, the average ESS score of our cohort was 4.86 (± 4.87), with only 21.4% of the children with PTLS with a score >10 (►Supplementary Tables 4 and 5, available in the online version). Furthermore, 71.4% of the children with PTLS did not take naps during the day (►Table 3). Thus, these results

Table 1 Parent-reported sleep concerns in children with PTLS

Sleep concern	Response frequency (%)				Respondents (n)
	Not during the past month	Less than once per week	Once or twice per week	Three or more times per week	
Cannot get to sleep within 30 min	21.4	0.0	28.6	50	14
Wake up in the middle of the night or early morning	21.4	7.1	21.4	50	14
Leg twitching/jerking in sleep	57.1	0.0	14.3	28.6	14
Cough or snore loudly	46.2	7.7	23.1	23.1	13
Feel too hot	76.9	0.0	0.0	23.1	13
Long pauses between breaths	42.9	14.3	21.4	21.4	14
Cannot breath comfortably	57.1	21.4	14.3	7.1	14
Trouble staying awake during activities	64.3	7.1	28.6	0.0	14
Feel too cold	83.3	8.3	8.3	0.0	12
Episodes of disorientation or confusion	64.3	7.1	28.6	0.0	14
Had bad dreams	69.2	30.8	0.0	0.0	13

Abbreviation: PTLS, Potocki–Lupski syndrome.

Table 2 Reported response frequency for PSQI components

PSQI component	Response frequency (%)				Respondents (n)
	Very good	Fairly good	Fairly bad	Very bad	
Component 1: sleep quality	23.1	38.5	38.5	0.0	13
	No phenotype	Mild	Moderate	Severe	
Component 2: sleep latency	0.0	28.6	35.7	35.7	14
Component 3: sleep duration	92.3	0.0	7.7	0.0	13
Component 4: sleep efficiency	69.2	15.4	7.7	7.7	13
Component 5: sleep disturbances ^a	10	60	30	0.0	10
	No medication	Less than one time per week	Once or twice per week	More than three times per week	
Component 6: sleep medication	57.1	0.0	0.0	42.9	14
	No phenotype	Mild	Moderate	Severe	
Component 7: daytime dysfunction	21.4	50	28.6	0.0	14

Abbreviation: PSQI, Pittsburgh Sleep Quality Index.

^aResponse frequency calculated from individuals that completed all requested items for this component.

indicate that the difficulty in maintaining the alert/awake state during the awake phase of the 24-hour sleep–wake cycle is not a major problem for most children with PTLS despite the presence of the nocturnal disturbance.³⁰ Overall, the majority of children with PTLS in our cohort have a composite sleep phenotype where the initiation and maintenance of sleep are disrupted resulting in a mild dyssomnia,

whereas other nighttime behavioral problems and daytime somnolence appear occasionally problematic.

Dysregulation of Circadian Genes in PTLS and *RAI1* Overexpression Cell Lines

Our survey data indicate that patients with PTLS have a composite sleep phenotype resulting in a mild degree of sleep disturbance. These data alone do not necessarily suggest a circadian rhythm defect. However, because haploinsufficiency of *RAI1* is shown to cause a dysregulation of core components of the circadian machinery, which results in sleep abnormalities,^{17,18} we sought to understand role of *RAI1* overexpression in the circadian machinery toward better understanding of the etiology behind the sleep disturbances observed. The core circadian machinery consists of two transcriptional activators, *CLOCK* and *ARNTL* (*BMAL1*), and two groups of transcriptional repressors, Periods (*PER*) and Cryptochromes (*CRY*). Therefore, we surveyed relative expression levels of these components in PTLS-derived LCLs. As a control, we tested the messenger RNA (mRNA) levels of *RAI1* within PTLS cells and found significantly elevated levels, which is consistent with duplication at 17p11.2 (►Fig. 1A) ($p < 0.0001$). Evaluation of circadian gene expression for the Period (*PER1–3*) family genes revealed significantly reduced transcript levels in PTLS LCLs (►Fig. 1A) ($p < 0.0001$). The Cryptochrome (*CRY1/2*) family mRNA levels in PTLS LCLs were unchanged relative to normal control cells ($p = 0.0910$ and $p = 0.0597$, respectively). These results indicate that the period of the circadian rhythms in individuals with PTLS may be compromised, but cryptochrome repression likely remains intact.

Since *CLOCK* and *ARNTL* were not highly expressed in LCLs (data not shown), we overexpressed *RAI1* in human embryonic kidney cells, HEK293T, and assayed gene expression at

Table 3 Prevalence of nighttime behaviors in children with PTLS

Behaviors	Yes (%)	No (%)	Respondents (n)
Difficult to wake	50	50	14
Use of sleep medication ^a	42.9	57.1	14
Child naps by themselves (no parental influence)	35.7	64.3	14
Arrangements for sleep containment	35.7	64.3	14
Child naps during the day	28.6	71.4	14
Difficult to put to bed	28.6	71.4	14
Seeks food at night	15.4	84.6	13
Wanders at night	7.1	92.9	14
Same problems napping as sleeping	0.0	100	13
Sleep walking	0.0	100	14

Abbreviation: PTLS, Potocki–Lupski syndrome.

^aMelatonin as the common sleep aid; clonidine in one case.

Fig. 1 Downstream targets of circadian locomotor output cycles *kaput* (*CLOCK*) have altered gene expression in Potocki–Lupski syndrome (PTLS) cells and *Retinoic acid induced 1* (*RAI1*) overexpression cell lines. (A) Circadian gene expression in PTLs patient’s lymphoblastoid cell lines (LCLs; black/gray bars) is altered compared with control LCLs (white bars). *PER1–3* are significantly reduced in PTLs patient-derived LCLs when compared with control cells, whereas *CRY1–2* were unchanged. (B) *CLOCK* and *ARNTL* messenger ribonucleic acid expression levels are not altered in HEK293T cells treated for overexpression of *RAI1* (black bars), whereas *PER1–3* and *CRY1–2* gene expression is altered in comparison to HEK293T control cells (white bars). The quantification of the test gene is compared with that of the housekeeping gene *Gapdh*. Error bars represent the standard deviation from the mean. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$.

24 hours to model PTLs. *RAI1* mRNA levels were significantly elevated to 1.65 fold ($p < 0.001$) relative to vehicle control cells (►Fig. 1B). However, *CLOCK* and *ARNTL* mRNA levels remained unchanged (►Fig. 1B). *PER1–3* transcript levels in *RAI1* overexpressed HEK293T lines had significantly reduced expression levels similar to the expression seen in PTLs LCLs (►Fig. 1B). *CRY1/2* gene expression levels were also significantly reduced, which differed from the transcript levels seen in PTLs LCLs (►Fig. 1B). Overall, our analysis suggests that an increase in *RAI1* dosage may not alter the master transcriptional regulators of circadian rhythmicity consisting of *CLOCK* and *ARNTL*; however, reduced *PER1–3* and *CRY1–2* expression may indicate disruption in circadian oscillations. Furthermore, different expression levels in *CRY1–2* in LCLs and HEK293T might suggest tissue-specific dysregulation of circadian genes.

Dysregulation of Circadian Genes in *Rai1-Tg* Hypothalamus during Light and Dark Phases

Patients with PTLs present with very mild degree of sleep disturbance (►Tables 1 and 2), and PTLs patient-derived LCLs show reduced mRNA levels of the Period (*PER1–3*) and Cryptochrome (*CRY1/2*) family genes (►Fig. 1A).^{6,16,31} The central clock, located in the suprachiasmatic nucleus (SCN) within the hypothalamus, is responsible for controlling central circadian rhythms and is entrained by external timing cues including photic environmental signals (light: dark).³² To investigate the role of *Rai1* overexpression on circadian machinery, we analyzed the mRNA levels of the circadian components within the hypothalamus of *Rai1-Tg* mice during both the light phase (~8 hours Zeitgeber time) and the dark phase (~16 hours Zeitgeber time). We identified a significant reduction of *Clock* ($p < 0.0001$) and *Arntl* ($p < 0.01$) transcript levels in both light and dark phases (►Fig. 2A, B). Furthermore, data show that mRNA levels of downstream targets of *Clock* and *Arntl*, including *Per1–3* and *Cry1/Cry2*, were also significantly reduced in the hypothalamus during the light phase in *Rai1-Tg* mice

Fig. 2 Altered expression of circadian genes in *Rai1-Tg* mouse hypothalamus during light and dark phases. (A) Circadian gene expression in mouse hypothalamus extracted from nontransgenic (NT; white bars) or *Rai1-Tg* (black bars) during the light phase at 8 hours Zeitgeber time. (B) Circadian gene expression in mouse hypothalamus extracted from NT (white bars) or *Rai1-Tg* mice (black bars) during the dark phase at 16 hours Zeitgeber time. The quantification of the test gene was compared with that of the housekeeping gene *Gapdh*. All expression data were confirmed from 3 to 6 mice of each genotype for each specific gene. Graphs represent mean $\pm$ standard error of mean, $n \geq 3$. ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$.

compared with the NT mice ($p < 0.05$) (►Fig. 2A). *Per1–3* and *Cry1/Cry2* in the dark phase were also dysregulated, mimicking the gene expression pattern observed in the light phase ($p < 0.0001$) (►Fig. 2B). Taken together, our gene expression analysis demonstrates that essential genes involved in circadian rhythmicity are dysregulated, suggesting SCN impairment.

Discordant Peripheral Clock Gene Expression in *Rai1*-Tg Mouse Heart, Liver, and Kidney Tissues

A compromised or altered SCN can lead to the abolishment of the circadian expression of core circadian machinery in peripheral tissues, leading to an imbalance of the homeostatic environment of circadian, metabolic, and sleep processes.³³ *Clock* gene function is essential to daily rhythms in heart energy metabolism,³⁴ and defects in *Clock* function can inhibit metabolic rhythms and cardiac function.³⁵ Since *Rai1*-Tg mice exhibit alterations of the light/dark phase entrainment of circadian gene expression within the hypothalamus, we predicted that peripheral tissues would likely mimic this failed entrainment.

Gene expression analysis revealed that *Rai1* transcript levels were overexpressed in all peripheral tissues studied (heart, liver, and kidney) in both light and dark phases (►Fig. 3A–F). In the light phase, *Clock* and all its downstream target genes are not significantly reduced in *Rai1*-Tg mice compared with NT mice in heart tissue (►Fig. 3A). However, in the dark phase, while normal levels of *Clock* were

observed, two circadian components, *Arntl* ($p < 0.0001$) and *Cry1* ($p < 0.05$), were significantly upregulated (►Fig. 3D). Another peripheral tissue essential to metabolism is the liver, where *Clock* gene expression is rhythmic.³⁶ Transcript levels were not significantly different between *Rai1*-Tg and NT mice in both light and dark phases (►Fig. 3B, E). In the light phase, transcript levels of the other circadian components were unchanged (►Fig. 3B); however, in the dark phase, *Arntl* ($p < 0.0001$), *Nr1d1* ($p < 0.01$), and *Per2* ($p < 0.0001$) were significantly upregulated (►Fig. 3E).

Most renal functions exhibit circadian oscillations,²⁵ and we examined the circadian gene expression across light and dark phases within *Rai1*-Tg kidney tissue. We found in both light and dark phases that *Clock* mRNA levels in *Rai1*-Tg were not significantly different from NT kidney (►Fig. 3C, F). However, *Arntl* expression was significantly upregulated in the light phase ($p < 0.0001$) but not in the dark phase (►Fig. 3C, F). In the dark phase, *Per2* expression levels were significantly upregulated ($p < 0.0001$), whereas *Nr1d1* levels were significantly reduced ($p < 0.001$) in

Fig. 3 Peripheral clock gene expression in *Rai1*-Tg mouse heart, liver, and kidney during light and dark phases is discordant with the central clock. Circadian gene expression in mouse heart (A,D), liver (B,E), kidney (C,F) tissue extracted from nontransgenic (white bars) or *Rai1*-Tg (black bars) during the light phase at 8 hours Zeitgeber time and during the dark phase at 16 hours Zeitgeber time. The quantification of the test gene was compared with that of the housekeeping gene *Gapdh*. Error bars represent the standard deviation from the mean, $n \geq 3$. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$.

Rai1-Tg compared with NT mice (– Fig. 3F). The gene expression changes observed in the dark phase are not consistent across each tissue. Mice are active during the dark phase and were allowed access to food and water. The few expression changes we did observe within the dark phase may be attributed to altered food intake, which is known to modulate circadian gene expression in the peripheral tissues.³⁷ Overall, our analysis of circadian machinery expression across the heart, liver, and kidney tissues of *Rai1*-Tg mice in light and dark phases revealed normal levels of *Clock* and the majority of its downstream targets; however, expression patterns were discordant with those observed in the hypothalamus.

Discussion

Our cross-translational approach to phenotypic characterization of observed sleep features in PTLs describes a mild sleep dyssomnia that is supported by the analysis of PTLs patient-derived LCLs, and expression studies in a *Rai1*-Tg PTLs mouse model that revealed a circadian deficiency. These two results provide a first look into sleep deficits and circadian dysfunction associated with *RAI1* overexpression.

Our sleep survey analysis, albeit limited in sample size, uncovered a composite sleep phenotype in children with PTLs consisting of a delayed onset of sleep initiation and a combination of both nocturnal and early morning awakenings, reaching DSM-V (Diagnostic and Statistical Manual of Mental Disorders, 5th Edition) diagnostic criteria indicative of a form of sleep dyssomnia.³⁸ The PSQI data presented here revealed significant concerns with nighttime awakenings as well as difficulty in initiating sleep, similar to that observed in children with ASD.³⁹ A previous 24-hour sleep study in a small subset of patients with PTLs with ASD also documented multiple nocturnal awakenings and mixed sleep apnea characterized by significant oxygen desaturation and hypercapnia, further supporting the diagnosis of a form of sleep dyssomnia.¹¹ Further analysis is warranted to elucidate whether circadian deficits are causal to sleep phenotype manifestations in these disorders. Our parent-reported survey analysis of the PTLs cohort had some limitations beyond the low sample size in assessing sleep problems, including the use of sleep aid medications, containment methods to limit nighttime behavioral problems, and the inability to normalize for a regimented sleep/nap routine. Considering these limitations, transitioning to studies in the *Rai1*-Tg mouse model was necessary, as the mice are more uniformly treated, without sleep aids, and are equally treated in day-to-day activity, allowing for a more consistent observation of core circadian changes based on genotype alone.

The circadian rhythms of the master clock depend on the autoregulatory transcription–translation loop of the circadian genes located in the SCN of the hypothalamus: *CLOCK*, *ARNT1*, *PER1–3*, and *CRY1/CRY2*. These components comprise the core clock machinery and generate 24-hour rhythms of gene expression.³² Circadian rhythms are adaptive to environmental cues, and entrainment of SCN clock also results in

peripheral clock entrainment through a variety of hierarchical pathways and networks.⁴⁰ Our analysis has shown that in the hypothalamus of *Rai1*-Tg mice, mRNA levels of *Clock*, *Arnt1*, *Per1–3*, and *Cry1/Cry2* are all significantly reduced in both light and dark phases, suggesting a partially failed photic signal entrainment of the circadian clock; however, surprisingly, the components of the circadian machinery in the peripheral tissues were largely unchanged in *Rai1*-Tg mice in both light and dark periods. The lack of circadian entrainment between the master clock within the hypothalamus and the peripheral tissues suggests that circadian dysregulation effects are likely more localized to the hypothalamus and other brain regions indicative of asynchronized rhythmicity.

The disconnect between the hypothalamus and the peripheral tissues is intriguing and would suggest aberrant communication promoting asynchronous expression patterns. One parsimonious explanation of the disconnection may be that defects in the hypothalamus remain localized to the SCN, a particular subset of other hypothalamic regions, and/or other brain regions, whereas the variety of hierarchical networks and pathways responsible for peripheral entrainment are only mildly affected, resulting in the uncoupling of core circadian components. Altered circadian gene expression within the hypothalamus commonly results in compromised metabolism and homeostatic energy functions such as impaired appetite and metabolic gene expression patterns.⁴⁰ This concept can be further supported by anthropometric and metabolic measures of *Clock*^{Δ19} and *Bmal1*^{–/–} mutant mice. Both mice display altered daily rhythm in feedings, leading to hyperphagia and subsequently altered lipids, leptin, glucose, and insulin levels.³² Analysis of various PTLs mouse models resulted in reduced weight, leaner body composition, a lower total cholesterol/low-density lipoprotein (LDL) ratio, and increased insulin sensitivity, whereas SMS mouse models with *Rai1* haploinsufficiency display increased weight, higher fat content, decreased high-density lipoprotein (HDL) levels, and reduced insulin sensitivity, suggesting that increased *RAI1* dosage may confer protection to anthropometric and metabolic features.^{6,25} Furthermore, patients with PTLs have significantly lower weight for age and length, and body mass index for age relative to a comparative reference population, whereas 90% of SMS patients by the age of 14 are at the 90th percentile for weight and commonly display hypercholesterolemia.^{14,25} Future directions dissecting the hierarchical networks and pathways between the hypothalamus and peripheral tissues that are affected by *RAI1* dosage may be an area of interest for identification of important therapeutic targets in these populations.

In summary, children with PTLs have mild sleep problems that include a prolonged onset of sleep initiation and maintenance of sleep. Components of the circadian machinery are dysregulated across the hypothalamus of *Rai1*-Tg mice in response to two different environmental cues (light/dark), whereas the peripheral tissue *Clock* expression remains unaffected. Hypothalamic-governed energy controls appear unaffected despite the asynchronous circadian expression

patterns between the hypothalamus and the peripheral tissues. Overall, our data demonstrate that overexpression of *RAI1* leads to dysregulation of proper gene expression within the circadian machinery as well as sleep deficiencies in individuals with PTLs.

Conflict of Interest

None.

Acknowledgments

The authors are appreciative to the families who participated in our study. The authors thank Coriell Cell Repositories for providing cell lines. The authors thank Kristi Schmidt and Meghan MacNeal for assistance with mice maintenance, breeding, and collection of mouse tissue. The authors thank the Smith–Magenis Syndrome Research Foundation, Virginia Commonwealth University, Baylor College of Medicine, Jan and Dan Duncan Neurological Research Institute, and Texas Children's Hospital for supporting this work. The National Science Foundation China grant (31200937) and Shanghai Health and Family Planning Commission grant (20144Y0106) funded L. C.

References

- Park SS, Stankiewicz P, Bi W, et al. Structure and evolution of the Smith–Magenis syndrome repeat gene clusters, SMS-REPs. *Genome Res* 2002;12(5):729–738
- Potocki L, Chen KS, Park SS, et al. Molecular mechanism for duplication 17p11.2– the homologous recombination reciprocal of the Smith–Magenis microdeletion. *Nat Genet* 2000;24(1):84–87
- Chen KS, Manian P, Koeuth T, et al. Homologous recombination of a flanking repeat gene cluster is a mechanism for a common contiguous gene deletion syndrome. *Nat Genet* 1997;17(2):154–163
- Elsea SH, Girirajan S. Smith–Magenis syndrome. *Eur J Hum Genet* 2008;16(4):412–421
- Elsea SH, Williams SR. Smith–Magenis syndrome: haploinsufficiency of *RAI1* results in altered gene regulation in neurological and metabolic pathways. *Expert Rev Mol Med* 2011;13:e14. Doi: 10.1017/S1462399411001827
- Girirajan S, Patel N, Slager RE, et al. How much is too much? Phenotypic consequences of *Rai1* overexpression in mice. *Eur J Hum Genet* 2008;16(8):941–954
- Treadwell-Deering DE, Powell MP, Potocki L. Cognitive and behavioral characterization of the Potocki–Lupski syndrome (duplication 17p11.2). *J Dev Behav Pediatr* 2010;31(2):137–143
- Gulhan Ercan-Sencicek A, Davis Wright NR, Frost SJ, et al. Searching for Potocki–Lupski syndrome phenotype: a patient with language impairment and no autism. *Brain Dev* 2012;34(8):700–703
- Lee CG, Park SJ, Yim SY, Sohn YB. Clinical and cytogenetic features of a Potocki–Lupski syndrome with the shortest 0.25Mb microduplication in 17p11.2 including *RAI1*. *Brain Dev* 2013;35(7):681–685
- Magoulas PL, Liu P, Gelowani V, et al. Inherited dup(17)(p11.2p11.2): expanding the phenotype of the Potocki–Lupski syndrome. *Am J Med Genet A* 2014;164A(2):500–504
- Potocki L, Bi W, Treadwell-Deering D, et al. Characterization of Potocki–Lupski syndrome (dup(17)(p11.2p11.2)) and delineation of a dosage-sensitive critical interval that can convey an autism phenotype. *Am J Hum Genet* 2007;80(4):633–649
- Ricard G, Molina J, Chrast J, et al. Phenotypic consequences of copy number variation: insights from Smith–Magenis and Potocki–Lupski syndrome mouse models. *PLoS Biol* 2010;8(11):e1000543. Doi: 10.1371/journal.pbio.1000543
- Yusupov R, Roberts AE, Lacro RV, Sandstrom M, Ligon AH. Potocki–Lupski syndrome: an inherited dup(17)(p11.2p11.2) with hypoplastic left heart. *Am J Med Genet A* 2011;155A(2):367–371
- Soler-Alfonso C, Motil KJ, Turk CL, et al. Potocki–Lupski syndrome: a microduplication syndrome associated with oropharyngeal dysphagia and failure to thrive. *J Pediatr* 2011;158(4):655–659.e2
- Jefferies JL, Pignatelli RH, Martinez HR, et al. Cardiovascular findings in duplication 17p11.2 syndrome. *Genet Med* 2012;14(1):90–94
- Girirajan S, Elsea SH. Abnormal maternal behavior, altered sociability, and impaired serotonin metabolism in *Rai1*-transgenic mice. *Mamm Genome* 2009;20(4):247–255
- Williams SR, Zies D, Mullegama SV, Grotewiel MS, Elsea SH. Smith–Magenis syndrome results in disruption of *CLOCK* gene transcription and reveals an integral role for *RAI1* in the maintenance of circadian rhythmicity. *Am J Hum Genet* 2012;90(6):941–949
- Mullegama SV, Pugliesi L, Burns B, et al. *MBD5* haploinsufficiency is associated with sleep disturbance and disrupts circadian pathways common to Smith–Magenis and fragile X syndromes. *Eur J Hum Genet* 2015;23(6):781–789
- Buyse DJ, Hall ML, Strollo PJ, et al. Relationships between the Pittsburgh Sleep Quality Index (PSQI), Epworth Sleepiness Scale (ESS), and clinical/polysomnographic measures in a community sample. *J Clin Sleep Med* 2008;4(6):563–571
- Buyse DJ, Reynolds CF III, Monk TH, Berman SR, Kupfer DJ. The Pittsburgh Sleep Quality Index: a new instrument for psychiatric practice and research. *Psychiatry Res* 1989;28(2):193–213
- Buyse DJ, Reynolds CF III, Monk TH, Hoch CC, Yeager AL, Kupfer DJ. Quantification of subjective sleep quality in healthy elderly men and women using the Pittsburgh Sleep Quality Index (PSQI). *Sleep* 1991;14(4):331–338
- Johns MW. A new method for measuring daytime sleepiness: the Epworth sleepiness scale. *Sleep* 1991;14(6):540–545
- Nishiyama T, Mizuno T, Kojima M, et al. Criterion validity of the Pittsburgh Sleep Quality Index and Epworth Sleepiness Scale for the diagnosis of sleep disorders. *Sleep Med* 2014;15(4):422–429
- Williams SR, Mullegama SV, Rosenfeld JA, et al. Haploinsufficiency of *MBD5* associated with a syndrome involving microcephaly, intellectual disabilities, severe speech impairment, and seizures. *Eur J Hum Genet* 2010;18(4):436–441
- Burns B, Schmidt K, Williams SR, Kim S, Girirajan S, Elsea SH. *Rai1* haploinsufficiency causes reduced *Bdnf* expression resulting in hyperphagia, obesity and altered fat distribution in mice and humans with no evidence of metabolic syndrome. *Hum Mol Genet* 2010;19(20):4026–4042
- Girirajan S, Elsea SH. Distorted Mendelian transmission as a function of genetic background in *Rai1*-haploinsufficient mice. *Eur J Med Genet* 2009;52(4):224–228
- Yan J, Bi W, Lupski JR. Penetrance of craniofacial anomalies in mouse models of Smith–Magenis syndrome is modified by genomic sequence surrounding *Rai1*: not all null alleles are alike. *Am J Hum Genet* 2007;80(3):518–525
- Bi W, Yan J, Shi X, et al. *Rai1* deficiency in mice causes learning impairment and motor dysfunction, whereas *Rai1* heterozygous mice display minimal behavioral phenotypes. *Hum Mol Genet* 2007;16(15):1802–1813
- Kendzierska TB, Smith PM, Brignardello-Petersen R, Leung RS, Tomlinson GA. Evaluation of the measurement properties of the Epworth sleepiness scale: a systematic review. *Sleep Med Rev* 2014;18(4):321–331

- 30 Young TB. Epidemiology of daytime sleepiness: definitions, symptomatology, and prevalence. *J Clin Psychiatry* 2004;65 (Suppl 16):12–16
- 31 Walz K, Paylor R, Yan J, Bi W, Lupski JR. Rai1 duplication causes physical and behavioral phenotypes in a mouse model of dup(17) (p11.2p11.2). *J Clin Invest* 2006;116(11):3035–3041
- 32 Silver R, Kriegsfeld LJ. Circadian rhythms have broad implications for understanding brain and behavior. *Eur J Neurosci* 2014;39(11):1866–1880
- 33 Franken P, Dijk DJ. Circadian clock genes and sleep homeostasis. *Eur J Neurosci* 2009;29(9):1820–1829
- 34 Kohsaka A, Das P, Hashimoto I, et al. The circadian clock maintains cardiac function by regulating mitochondrial metabolism in mice. *PLoS One* 2014;9(11):e112811. Doi: 10.1371/journal.pone.0112811
- 35 Lefta M, Campbell KS, Feng HZ, Jin JP, Esser KA. Development of dilated cardiomyopathy in Bmal1-deficient mice. *Am J Physiol Heart Circ Physiol* 2012;303(4):H475–H485
- 36 Rui L. Energy metabolism in the liver. *Compr Physiol* 2014;4(1):177–197
- 37 Satoh Y, Kawai H, Kudo N, Kawashima Y, Mitsumoto A. Time-restricted feeding entrains daily rhythms of energy metabolism in mice. *Am J Physiol Regul Integr Comp Physiol* 2006;290(5):R1276–R1283
- 38 American Psychiatric Association. Diagnostic and Statistical Manual of Mental Disorders. 5th ed. Washington, DC: American Psychiatric Association; 2013
- 39 Veatch OJ, Maxwell-Horn AC, Malow BA. Sleep in autism spectrum disorders. *Curr Sleep Med Rep* 2015;1(2):131–140
- 40 Froy O. Metabolism and circadian rhythms—implications for obesity. *Endocr Rev* 2010;31(1):1–24