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Research Letter

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Novel *CLOCK* and *NR1D2* variants in 64 sighted Japanese individuals with non-24-hour sleep–wake rhythm disorder

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Circadian rhythm sleep–wake disorders (CRSWDs) are characterized by an inability to fall asleep and awaken at desired times. A subtype of CRSWDs, non-24-hour sleep–wake rhythm disorder (N24SWD), exhibits a free-running pattern of sleep–wake cycles that are not synchronized with the external 24-hour day. N24SWD occurs commonly in visually impaired individuals and rarely in sighted individuals. We have shown that the intrinsic circadian period (τ) determined under a forced desynchrony protocol is longer in sighted individuals with N24SWD than intermediate-type controls [1]. Therefore, it appears that prolonged τ and/or impaired entrainment mechanisms contribute to the pathogenesis of N24SWD. Some clock gene variants are associated with CRSWDs and transgenic animals carrying human clock variants exhibit altered τ [2]. These findings suggest that clock gene variants might disrupt the circadian clock system and lead to the onset of CRSWDs.

The study population consisted of 64 participants with N24SWD (45 males and 19 females; mean ± SD age: 27.7 ± 9.61 years). Most of the participants were examined in our previous studies [1, 3]. They were all unrelated and sighted Japanese. They were diagnosed by trained psychiatrists according to the International Classification of Sleep Disorders' second edition. This study was approved by the Ethics Committee of National Center of Neurology and Psychiatry and was conducted in accordance with the declaration of Helsinki. Written informed consent was obtained from each participant. DNA samples were extracted from the participants' blood samples using the QIAamp DNA Blood Mini Kit (QIAGEN). Targeted sequencing of 76 genes was performed in 17 individuals with N24SWD (11 males and six females; mean ± SD age: 32.82 ± 10.05 years) using next-generation sequencing by RIKEN GENESIS (RIKEN GENESIS CO., LTD.). Briefly, DNA samples were captured using the SureSelect DNA Capture Custom Kit (Agilent Technologies) and sequenced on the MiSeq system (Illumina) with 151 bp paired-end reads. The reads were aligned to a human reference sequence (University of California Santa Cruz assembly GRCh37/hg19). The 76 genes examined in this study are listed in Supplementary Table 1. Sanger sequencing was performed using BigDye™ Terminator v1.1 Ready Reaction Mix (ThermoFisher Scientific) and an ABI Prism 3130 DNA Analyzer (Applied Biosystems). All primers were designed by Primer3Plus. The possible impact of amino acid substitutions on protein function was tested by Polyphen-2 and PROVEAN (Protein Variation Effect Analyzer).

In this study, we performed targeted sequencing of 76 genes by next-generation sequencing in 17 N24SWD individuals and found a total of 94 variants (Supplementary Table 2). A novel missense variant was initially found in each of the *APOE* (*apolipoprotein E*), *CLOCK* (*CLOCK*), *NR1D2* (*nuclear receptor subfamily 1 group D member 2*), and *PER1* genes The *NR1D2* (PERIOD1) and *PER1* missense variants were found in an individual with N24SWD. These four variants were further evaluated by public databases, the Genome Aggregation Database, 1000 Genomes and Human Genetic Variation Database, and the Japanese Whole Genome Reference Panel 8.3KJPN. The *APOE* and *CLOCK* variants (designated as rs1969838696 and rs1723064872, respectively) were recently observed as rare variants in 8.3KJPN (alternative allele frequency = 0.00012), while the *NR1D2* and *PER1* variants were not found in any database. Previous studies using animal models have suggested that *Clock* and *REV-ERBβ* (*NR1D2*) are involved in some mechanisms that regulate circadian rhythms and

sleep–wakefulness [2, 4, 5]. In contrast, *APOE*-deficient and *Per1*-null mice show a robust behavioral rhythm comparable to that of WT mice [6, 7]. Therefore, our initial focus was on the *CLOCK* and *NR1D2* genes, which have been implicated as core components of negative transcriptional feedback loops regulating multiple clock genes in the circadian clock system [2, 4, 5]. We subsequently performed Sanger sequencing on the coding regions of *CLOCK* and *NR1D2* in a total of 64 N24SWD individuals, including 17 N24SWD individuals.

One novel and four known *CLOCK* variants were identified in our study population of 64 N24SWD individuals (Supplementary Table 3). A novel variant in exon 18 was identified in one of the additional 47 N24SWD individuals (NM_004898.3: c.1488C>G: p.Q[Gln]496H[His]) (Figure 1A). The amino acids Q and H differ in isoelectric point and the Q496H substitution is predicted to be possibly damaging (0.887) by Polyphen-2 and deleterious (–2.854) by PROVEAN. The Q496H substitution occurs in the domain that potentially associates with SIRT1, a NAD+-dependent histone deacetylase. SIRT1 is recruited to the CLOCK:BMAL1 chromatin complex and regulates target gene transcription by modulating the histone acetyltransferase function of CLOCK [8]. The Q496H substitution could alter the binding interaction between CLOCK and SIRT1 thereby disrupting chromatin remodeling. The known variant in exon 22 (rs1723064872) causes the amino acid substitution of glutamine to glutamate (NM_004898.3: c.2278C>G: p.Q[Gln]760E[Glu]). The amino acids Q and E differ in isoelectric point, although the Q760E substitution is predicted to be benign (0.033) by Polyphen-2 and neutral (–1.289) by PROVEAN. The Q760E substitution is located in the poly-Q region of the C-terminal domain. The Q-rich motif is known to characterize the activation domain of transcription factors. Furthermore, *Clock* mutant mice show a longer period of behavioral rhythms than wild-type mice. The genetic variant carried by *Clock* mutants results in exon skipping and a deletion of 51 amino acids within the CLOCK transactivation domain [4]. Notably, the missense variants in the potentially functional domains of CLOCK were identified in two N24SWD individuals. These *CLOCK* missense variants might contribute to the N24SWD phenotype.

One novel and eight known *NR1D* variants were identified in our study population of 64 N24SWD individuals (Supplementary Table 3). The novel variant in exon 2 causes an amino acid substitution of glycine to serine (NM_005126.4: c.274G>A: p.G[Gly]92S[Ser]) (Figure 1B). The amino acids G and S differ in polarity, although the G92S substitution is predicted to be benign (0.011) by Polyphen-2 and neutral (-1.094) by PROVEAN. A known variant in exon 5 (rs139583758) causes the amino acid substitution of glutamine to histidine (NM_005126.4: c.696A>C: p.Q[Gln]232H[His]). The amino acids Q and H differ in isoelectric point and the Q232H substitution is predicted to be possibly damaging (0.925) by Polyphen-2 and be deleterious (–2.518) by PROVEAN. Another known variant in exon 7 (rs78292562) causes the amino acid substitution of alanine to threonine (NM_005126.4: c.1351G>A: p.A[Ala]451T[Thr]). Amino acids A and T differ in polarity. Furthermore, the A451T substitution is predicted to be probably damaging (0.995) by Polyphen-2 and deleterious (–3.157) by PROVEAN. The A451T substitution occurs in the potential ligand-binding domain of NR1D2. Heme binds to the ligand-binding domain and modulates the ability of NR1D2 to recruit the corepressor and repress target gene transcription [9]. NR1D1 (REV-ERBα) and NR1D2 (REV-ERBβ) regulate sleep architecture and emotional behavior in mice [5]. REV-ERB agonists induce wakefulness and reduce rapid eye movement and slow-wave sleep. Intriguingly, a pharmacological study in REV-ERBβ-deficient mice suggests that REV-ERBβ modulates the maintenance of wakefulness during the activity period [10]. These *NR1D2* missense variants could alter the function of NR1D2, resulting in impaired sleep regulation. Also, the novel *PER1* variant was found in the N24SWD individual carrying the novel *NR1D2* variant in exon 2. The *PER1* variant in exon 10 (NM_002616.2: c.1198G>A: p.E[Glu]400K[Lys]) was confirmed by Sanger sequencing in the N24SWD individual (Figure 1C).

Further analysis is required to demonstrate that these variants contribute to the N24SWD phenotype. However, our findings will provide potential genetic factors associated with the N24SWD phenotype and expand the current understanding of circadian and sleep regulation in humans.

Supplementary Material

Supplementary material is available at *SLEEP* online.

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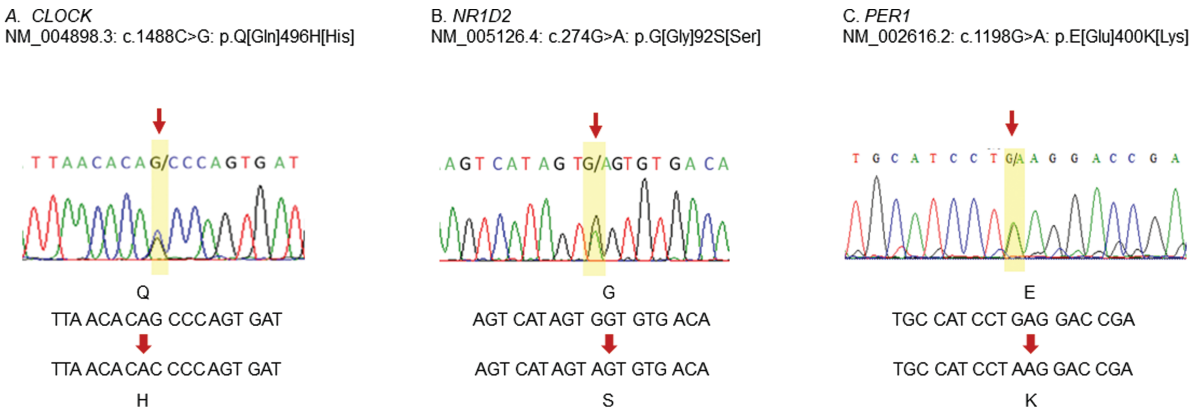

Figure 1. Novel missense variants of the CLOCK, *NR1D2*, and *PER1* genes in N24SWD individuals. Sanger sequencing confirms a novel missense variant in each of *CLOCK* (A), *NR1D2* (B) and *PER1* (C) as indicated by an arrow.

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Disclosure Statement

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Data Availability

The data underlying this article are available in the article and in its online supplementary material.

References

1. Kitamura S, *et al*. Intrinsic circadian period of sighted patients with circadian rhythm sleep disorder, free-running type. *Biol Psychiatry*. 2013;**73**(1):63–69. doi: 10.1016/j.biopsych.2012.06.027
2. Takahashi JS, *et al*. The genetics of mammalian circadian order and disorder: implications for physiology and disease. *Nat Rev Gene*. 2008;**9**(10):764–775. doi: 10.1038/nrg2430
3. Hida A, *et al*. Screening of clock gene polymorphisms demonstrates association of a *PER3* polymorphism with morningness-eveningness preference and circadian rhythm sleep disorder. *Sci Rep*. 2014;**4**:6309. doi: 10.1038/srep06309
4. King DP, *et al*. Positional cloning of the mouse circadian *clock* gene. *Cell*. 1997;**89**(4):641–653. doi: 10.1016/s0092-8674(00)80245-7
5. Banerjee S, *et al*. Pharmacological targeting of the mammalian clock regulates sleep architecture and emotional behaviour. *Nat Commun*. 2014;**5**:5759. doi: 10.1038/ncomms6759
6. Chalfant JM, *et al*. Circadian disruption with constant light exposure exacerbates atherosclerosis in male *ApolipoproteinE*-deficient mice. *Sci Rep*. 2020;**10**(1):9920. doi: 10.1038/s41598-020-66834-9
7. Takasu NN, *et al*. In vivo monitoring of multi-unit neural activity in the suprachiasmatic nucleus reveals robust circadian rhythms in *Period1*$^{-/-}$ mice. *PLoS One*. 2013;**8**(5):e64333. doi: 10.1371/journal.pone.0064333
8. Nakahata Y, *et al*. The NAD+-dependent deacetylase SIRT1 modulates CLOCK-mediated chromatin remodeling and circadian control. *Cell*. 2008;**134**(2):329–340. doi: 10.1016/j.cell.2008.07.002
9. Burris TP. Nuclear hormone receptors for heme: REV-ERBalpha and REV-ERBbeta are ligand-regulated components of the mammalian clock. *Mol Endocrinol*. 2008;**22**(7):1509–1520. doi: 10.1210/me.2007-0519
10. Amador A, *et al*. REV-ERBβ is required to maintain normal wakefulness and the wake-inducing effect of dual REV-ERB agonist SR9009. *Biochem Pharmacol*. 2018;**150**:1–8. doi: 10.1016/j.bcp.2018.01.009