A Sleep Bruxism Model using Neural Cells Derived from Patient-specific iPSCs with 5-HT2A Polymorphism

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Short title: A Cellular Model of Sleep Bruxism

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Abstract

In the second edition of the International Classification of Sleep Disorders (ICSD-2), sleep bruxism (SB) is classified as a sleep-related movement disorder characterized by grinding and clenching of the teeth during sleep, which is responsible for a variety of clinical problems such as temporomandibular disorders, abnormal tooth attrition, fracture of teeth or roots, and aggravation of periodontal diseases. Little is known about the etiology of SB; however, an involvement of genetic factors in SB was inferred from twin studies and other familial aggregation studies. Our previous study identified a genomic association of the serotonin 2A receptor (5-HT2A) single nucleotide polymorphism (SNP), rs6313 C>T, with SB, where the C allele carrier is associated with a 4.25-fold increased risk of SB. Based on this finding, the aim of this study was to establish a SB disease model using patient-specific induced pluripotent stem cells (iPSCs). Two SB patients with the risk allele (C/C genotype) of rs6313 and two controls without it (T/T genotype) were screened by laboratory-based polysomnographic recordings and the TaqMan genotyping assay. Four lines of iPSCs, two from SB patients and two from controls, were established from peripheral blood mononuclear cells by introduction of reprogramming factors. We performed quality control assays on iPSCs using expression of markers for undifferentiated pluripotent cells, immunostaining for
pluripotency markers, a three-germ layer assay, and karyotype analysis. The established iPSCs were differentiated into neurons using the neurosphere culture system. $5-HT2A$ gene expression in these neurons was evaluated by quantitative real-time PCR. Patient-specific iPSCs were successfully differentiated into neurons expressing $5-HT2A$. This report is the first successful development of a novel in vitro SB model, which has the potential to elucidate the etiology and underlying mechanism of SB.
Introduction

In the second edition of the International Classification of Sleep Disorders (ICSD-2), sleep bruxism (SB) is classified as a sleep-related movement disorder characterized by grinding and clenching of the teeth during sleep, which is observed in 3–8% of the adult population [1]. SB is responsible for a variety of pains and dysfunctional conditions in the orofacial region, such as temporomandibular disorders, abnormal tooth attrition, fracture of teeth or roots, and aggravation of periodontal diseases [2]. Furthermore, the mechanical stress of SB is associated with poor prognosis of dental treatment and seriously compromises patients’ quality of life. However, little is known about the etiology of SB and therefore treatment remains limited to symptomatic therapy, such as usage of a mouth guard to protect oro-dental structures from the force of SB.

It is clear from the literature that SB is regulated centrally rather than peripherally. More specifically, SB episodes occur as part of the partial arousal phenomena, which is associated with transient elevations of central and sympathetic nervous system activity during sleep [3,4], suggesting a possible involvement of brain neurotransmitters in the pathogenesis of SB. In fact, several studies reported associations between SB and central acting drugs. For example, the selective serotonin reuptake inhibitors prescribed for depression have been reported to cause SB [5,6], which can be
controlled successfully by the serotonin 1A receptor agonist [7]. In addition, the α2 adrenergic receptor agonist clonidine significantly reduced SB compared with a placebo, which was associated with reduction of sympathetic activity during sleep [8].

On one hand, an involvement of genetic factors in SB was inferred from twin studies [9] and other familial aggregation studies [10,11]. Previously, we found a genomic association of the serotonin 2A receptor (5-HT2A) single nucleotide polymorphism (SNP), rs6313 C>T, with SB, where the C allele carrier is associated with a 4.25-fold increased risk of SB [12]. The human 5-HT2A gene is located on chromosome 13 (13q14-21), consists of three exons separated by two introns, and spans approximately 20 kb in length [13]. 5-HT2A has been a molecule of particular interest in biological psychiatry because it is an important target for psychotropic drugs [14,15], and altered 5-HT2A expression has been found in several neuropsychiatric conditions, including depression [16] and schizophrenia [17].

Rs6313 in exon 2 and rs6311 in the upstream or promoter region of 5-HT2A are the most frequently cited SNPs in studies concerning 5-HT2A. They are in near-perfect linkage disequilibrium [18], located 1538 bases apart on chromosome 13, and neither alters the encoded protein. Genetic association has been reported between these polymorphisms and a number of clinical phenotypes,
including schizophrenia [19,20], psychotic symptoms in Alzheimer’s disease [21], certain features of depression [22], sleep breathing disorders [23], and also SB [12].

Understanding the functional consequences of genetic variants is a critical first step towards appreciating their roles in disease. A post-mortem study demonstrated that the C allele of rs6313 was related to reduced binding of 5-hydroxytryptamine to the 5-HT2A receptor in the superior frontal cortex compared with the T allele [24]. An in vitro study reported that the G allele of the rs6311 SNP, which is in absolute linkage disequilibrium with the C allele of rs6313, has the potential to negatively modulate 5-HT2A promoter activity [25]. Another post-mortem brain study reported that expression of 5-HT2A was higher in individuals carrying the C allele of rs6313 (or G allele of rs6311) than in individuals with the T/T genotype [26]. Electrophoretic mobility shift assay found allele-specific binding of E47 to the A allele but not the G allele of rs6311, which is associated with up-regulation of 5-HT2A gene expression in chronic fatigue syndrome [27]. These findings suggest that the implicated genetic variants have functional consequences. On the other hand, multiple studies failed to find direct associations of genetic variants with 5-HT2A gene expression [28-30], perhaps owing to environmental factors that alter 5-HT2A expression [31-34].

However, its underlying molecular mechanisms contributing to SB have not been
elucidated so far. Although our previous study found a significant association between SB and the rs6313 SNP, the effects of the SB-associated variant on 5-HT2A gene expression and the function of the serotonergic system have not been investigated, mainly because of the limited accessibility to the brain.

Previous explorations of human neurological and psychiatric disorders were hampered by the difficulty in obtaining patient-derived neural cells or tissues because of the limited accessibility to the brain. In addition, there are no appropriate animal models in which to study SB at present. In vitro construction of human disease models using disease-specific or patient-specific induced pluripotent stem cells (iPSCs) has the potential to provide dramatic progress in the elucidation of the pathogenetic mechanisms of such diseases [35-37]. Thus, we used disease-specific iPSC technology to reveal the pathogenic mechanism of SB. We here report the first successful development of a novel in vitro SB model and show 5-HT2A expression in neurons differentiated from SB patients’ iPSCs.

Materials & Methods
Subjects

Two primary SB patients [1] and two age-matched healthy controls were selected from the SB and control groups. These two groups were established based upon clinical diagnostic criteria, which was confirmed by two night polysomnography (PSG) recordings in a case-control study.

Clinical diagnosis of SB was made based on the following criteria: 1) reports of tooth-grinding sounds by their sleep partner; 2) presence of tooth attrition with exposed dentin; 3) reports of morning masticatory muscle fatigue or tenderness; and 4) presence of masseter muscle hypertrophy [38]. Then, PSG studies were conducted in these individuals in a sleep laboratory, and the sleep structure and SB episodes were analyzed. The following PSG-based SB diagnostic criteria were applied to each participant: 1) more than four bruxism episodes per hour; 2) more than six bruxism bursts per episode and/or 25 bruxism bursts per hour of sleep; and 3) at least two episodes with grinding sounds [39]. SB diagnosis of each participant was confirmed. Then, genomic DNA was extracted from whole blood of these subjects to determine the genotype of rs6313 in 5-HT2A using a fluorogenic TaqMan probe (Thermo Fisher Scientific) with quantitative real-time PCR (qRT-PCR). Our previous study identified the C allele of the rs6313 SNP as a risk allele for SB; therefore, we selected two SB subjects with the C/C genotype and two controls with the T/T
genotype from the above-mentioned groups.

**Generation of iPSCs**

iPSCs were generated from monocytes in peripheral blood samples of two SB patients and two controls by transducing episomal plasmids encoding transcription factors (OCT3/4, SOX2, KLF4, LIN28, and L-MYC) and dominant-negative p53 via electroporation, respectively, and three iPSC clones were isolated from each individual [40,41]. All iPSC clones were evaluated based on the expression of pluripotent markers, the disappearance of episomal transgenes, and *in vitro* differentiation assays. The cells were passaged once every 5–7 days.

All experimental procedures for iPSC production were approved by the ethics committee of the Showa University of Human Ethics Committee (approval no. 179) and the Keio University School of Medicine Ethics Committee (approval no. 20080016).

**Karyotyping and genotyping**

Standard G-banding analysis of iPSCs was performed to rule out the possibility of abnormal karyotypes that can occur during the generation of iPSCs. SNP analysis was conducted on genomic
DNA extracted from iPSCs to determine the genotype of rs6313 in 5-HT2A using a fluorogenic TaqMan probe with qRT-PCR.

In vitro differentiation

iPSCs were harvested by treatment with CTK dissociation solution and used for embryoid body formation. Clumps of cells were transferred to Petri dishes in DMEM/F12 containing 20% knockout serum replacement (Life Technologies), 2 mM L-glutamine, 0.1 mM nonessential amino acids, 0.1 mM 2-mercaptoethanol (Life Technologies), and 0.5% penicillin/streptomycin. The medium was changed every other day. For spontaneous differentiation, 8-day-old embryoid bodies were plated onto gelatin-coated coverslips and allowed to differentiate in DMEM containing 10% fetal bovine serum for an additional 8 days.

Neuronal differentiation of iPSCs

Neuronal induction of iPSCs was performed as previously described [42]. Briefly, iPSCs were pretreated for 6 days with 3 mM SB431542 (Tocris), 3 μM dorsomorphin, and 3 mM CHIR99021 (Stemgent). They were then dissociated and seeded at a density of 10 cells/ml in medium hormone
mix [43,44] with growth factors and inhibitors under conditions of 4% O$_2$/5% CO$_2$. The growth factors and inhibitors included 10 ng/ml human leukemia inhibitory factor (Nacalai Tesque), 20 ng/ml fibroblast growth factors-2, 1× B27 supplement (Invitrogen), 2 mM SB431542, 0.5 mM CHIR99021, and 10 mM Y-27632 (Calbiochem). Defining the day on which neurosphere culture was started as day 0, 100 ng/ml Shh-C24II (R&D Systems) and 1 µM purmorphamine (Calbiochem) were added to cultures on day 2. Neurospheres were replated en bloc on dishes coated with poly-ornithine and laminin, and cultured under conditions of 5% CO$_2$ on day 12. The medium was changed to medium hormone mix supplemented with 1× B27 and 1 µM DAPT (Sigma). Cells were analyzed on day 24.

**Immunocytochemistry of iPSCs**

Cells were fixed with 4% paraformaldehyde for 15 minutes at room temperature and then washed three times with PBS. The cells were incubated with primary antibodies against the following proteins: OCT4 (Monoclonal antibody, 1:200, Santa Cruz Biotechnology; sc-5279), TRA-1-81 (Monoclonal antibody, 1:1000, Millipore; MAB4381), SSEA4 (Monoclonal antibody, 1:1000, Millipore; MAB4304), AFP (Monoclonal antibody, 1:250, R&D; MAB1368), α SMA (Polyclonal
antibody, 1:150, abcam; ab5694), and βIII-tubulin (Monoclonal antibody, 1:250, Sigma Chemical; T8328). They were again washed three times with PBS and incubated with secondary antibodies conjugated with Alexa Fluor 488, Alexa Fluor 555, and Alexa Fluor 647, followed by Hoechst 33342 (Dojindo Laboratories) for 1 hour at room temperature. After washing three times with PBS and once with distilled water, samples were mounted on slides and examined using a VZ-9000 box-type microscope (Keyence) and a LSM-710 confocal laser-scanning microscope (Carl Zeiss).

**qRT-PCR**

RNA was isolated with an RNeasy mini kit (Qiagen), and first-strand cDNA was synthesized with iScript (Toyobo). qRT-PCR was performed using SYBR premix EX Taq II (Takara Bio) and analyzed with a ViiA7 real-time PCR system. The primers used to detect ACTB and 5-HT2A are summarized in Table 1. As a positive control we used human cortex cDNA (CLN, 639320). All values were expressed relative to ACTB expression. Reactions were carried out in triplicate, and data were analyzed using the comparative Ct method.

**Table 1. Primer details for RT-PCR**

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Results

Patient information

The demographic data and SB variables of the four subjects are summarized in Table 2. All of the subjects were Japanese. The SB patients exhibited moderate to severe SB levels and had the rs6313 risk allele for SB, while the control subjects exhibited no phasic episodes with grinding sounds and did not have the risk allele. The sleep structure of all subjects was within the normal range, and no other sleep disorders were identified by the PSG data.

<table>
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Table 2. Demographic data, SB data, and genotypes of the subjects
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<td>Episodes with sounds</td>
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<td>21</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
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<td>C / C</td>
<td>C / C</td>
<td>T / T</td>
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**Generation of SB and control iPSCs**

Four lines of iPSCs, two from SB patients and two from controls, were established from peripheral blood. In addition, the following iPSC clones were selected for detailed analysis: three from patient SB1 (SB1-1, SB1-2, and SB1-10), three from patient SB2 (SB2-2, SB2-7, and SB2-10), three from control 1 (C1-6, C1-12, and C1-18), and three from control 2 (C2-1, C2-2, and C2-4). All iPSC colonies had a typical human embryonic stem cell (ESC) morphology, with tightly packed cells, a clear border, and a round shape (Fig 1A, S1). Expression of human ESC markers (Fig 1A, S1) and differentiation capability into three germ layers (Fig 1C), which supports the undifferentiated state and pluripotency of iPSCs, respectively, were confirmed. No abnormalities were found in the
karyotypes of the iPSC lines (Fig 1B). SNP analysis using a fluorogenic TaqMan probe with qRT-PCR confirmed that the rs6313 genotype was preserved in the established iPSC lines (Fig 1D). Thus, the generated iPSC lines were successfully reprogrammed into a pluripotent state and were suitable for further analysis.

Figure 1. Characterization of generated iPSCs

(A) The morphology of all iPSC lines was similar to that of human ESCs. Both control and SB iPSCs expressed the human ESC markers OCT3/4, TRA1-81, and SSEA-4. Scale bar, 200 µm. (B) Standard G-banding analysis. Karyotype analysis revealed that iPSCs had a normal karyotype. (C) In vitro three-germ layer assay. Cells differentiated from iPSCs expressed an endoderm marker (AFP), a mesoderm marker (αSMA), and an ectoderm marker (βIII-tubulin). Scale bar, 100 µm. (D) SNP analysis using a fluorogenic TaqMan probe with qRT-PCR. The subjects were selected based not only on SB diagnosis, but also on their rs6313 genotype (SB, C/C; control, T/T). The rs6313 genotype of T cells of patient SB1 (♢ C/C) and control C1 (○ T/T) were preserved in SB1-1 iPSCs (♦ C/C) and C1-18 iPSCs (● T/T), respectively. NTC (■) was no template control.
Increased $5-HT2A$ levels in SB patient iPSC- and control iPSC-derived neurons

Since $5-HT2A$-positive neurons are located in the raphe nucleus in the ventral hindbrain [45]; therefore, we derived neurons with the characteristics of this brain region from SB-specific iPSCs using previously described procedures [42] (Fig 2A). The expression levels of $5-HT2A$ gene of these neurons were much higher than those of T cells raised from the original peripheral mononuclear cells with stimulation by IL-2 and anti-CD3 antibody and iPSCs (Figure 2B). These results indicate that $5-HT2A$-positive neurons, which are the cells responsible for SB, were successfully induced from SB-specific iPSCs.

Figure 2. Neurons express $5-HT2A$

(A) Schematic procedures for neural differentiation from iPSCs into neurons that express $5-HT2A$.

(B) The expression levels of $5-HT2A$ gene of these neurons were much higher than those of T cells raised from the original peripheral mononuclear cells with stimulation by IL-2 and anti-CD3 antibody and iPSCs. Data represent the mean of at least three experiments for each group. Samples were compared with T cells of patient SB1.
Discussion

SB patients present with a wide range of diverse clinical outcomes in oro-dental regions due to intense trigeminal motor activities during sleep. As described in the Introduction section, there is a consensus that SB episodes are associated with transient elevations of central and sympathetic nervous system activity during sleep [3,4], suggesting a possible involvement of brain neurotransmitters in the pathogenesis of SB. Besides, we found an association between genetic variation of 5-HT2A and SB [12]. However, explorations of functional associations between the serotonergic system and the pathogenesis of SB have been hampered by the difficulty in obtaining patient-derived neural cells because of the limited accessibility to the brain. The absence of clear knowledge regarding the pathogenesis and pathophysiology of human SB has been considered the major cause of the failure to develop logical therapeutic strategies. Indeed, SB treatment remains limited to symptomatic therapy, such as usage of a mouth guard to protect oro-dental structures from the force of SB [46]. However, reported associations between SB and central acting drugs [5-7] suggest that medication may be a promising method to treat SB patients, although it remains obscure which mechanisms are involved in the effects of these drugs.

Disease-specific iPSCs are available from patients with a variety of conditions, including
nervous system, hematopoietic system, and metabolic system diseases, and investigations of their pathologies are progressing rapidly [33,47-49]. The use of iPSCs derived from patients with certain neurological diseases permits the preparation of brain cells that contain the actual genetic information of the patients themselves. This is a notable feat given that such cells have been technologically and ethically difficult to obtain in the past [50]. In this study, we established SB-specific iPSCs and successfully differentiated 5-HT2A-expressing neurons that contain the genetic information of a SB patient. The subjects selected in this study exhibited moderate to severe SB levels with no comorbidity, which was confirmed by PSG recordings, and carried the risk allele (C allele of rs6313) for SB [12]. Although a substantial number of point mutations in iPSCs is reported to occur in a transversion-predominant manner during the conversion process, especially at the initiation steps, from somatic cells to iPSCs [51], genomic DNA of iPSCs derived from the SB patients and controls agreed with the original genotype, which indicated that iPSCs derived from both groups preserved the SNP rs6313 in 5-HT2A. Consequently, these processes ensured that the disease-specific iPSC model of primary SB possesses a genetic origin. The established SB patient-specific iPSCs can be applied to disease modeling, not only for better understanding of the pathogenesis of SB but also for drug screening for SB patients. In addition, gene correction of the
SB patient-specific iPSCs using current gene engineering technologies, such as CRISPR/CAS-9, a sequence-specific designed zinc finger nuclease, or a transcription activator-like effector nuclease for SB patient-specific iPSCs [52], is expected to clarify whether the 5-HT2A variants are causal factors.

In this study, we used a culture system that controlled the regional identity of iPSC-derived neurons along the antero-posterior and dorso-ventral axes [42]. By using this system, we derived neurons with the characteristics of the ventral hindbrain, in which 5-HT2A is expressed. Indeed, the 5-HT2A level was higher in iPSC-derived neurons than in T cells and iPSCs. These differentiated neurons expressing 5-HT2A could be identified by a fluorescent reporter under the control of the 5-HT2A promoter or by immunostaining techniques, which allows detailed and thorough analyses of the function and morphology of these neurons.

In addition, our method can elucidate the mechanism underlying other types of diseases that are associated with 5-HT2A polymorphisms, such as schizophrenia [19,20], psychotic symptoms in Alzheimer’s disease [21], certain features of depression [22], and sleep breathing disorders. For example, the functional consequences of genetic variants of rs6313, which is one of the most frequently studied 5-HT2A polymorphisms, remain controversial and are not fully understood.
[24-26,28,29], because the target cells are technologically and ethically difficult to obtain, as discussed above. The successful differentiation of neurons expressing 5-HT2A from patient-specific iPSCs opens up new avenues to elucidate the pathophysiological mechanisms of these human diseases.

**Acknowledgments**

The authors are grateful to S. Morimoto, M. Ishikawa for technical assistance and helpful advice. We also thank all members of the H.O. laboratory for encouragement and kind support. This study was supported by JSPS KAKENHI Grant Number 26393415.

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Supporting Information

S1 Fig. Characterization of generated iPSCs

The morphologies of the iPSC lines SB1-2, SB1-10, SB2-2, SB2-7, SB2-10, C1-6, C1-12, C2-1, C2-2, and C2-4 were similar to that of human ESCs. iPSCs expressed the human ESC markers OCT3/4, TRA1-81, and SSEA-4. Scale bar, 200 µm.