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Hypothalamic orexin and mechanistic target of rapamycin activation mediate sleep dysfunction in a mouse model of tuberous sclerosis complex

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A B S T R A C T

Tuberous sclerosis complex (TSC) is a genetic disease related to hyperactivation of the mechanistic target of rapamycin (mTOR) pathway and manifested by neurological symptoms, such as epilepsy and sleep disorders. The pathophysiology of sleep dysfunction is poorly understood and is likely multifactorial, but may involve intrinsic biological regulators in the brain. Here, we characterized a mouse model of sleep disorders in TSC and investigated mechanisms of sleep dysfunction in this conditional knockout model involving inactivation of the Tsc1 gene in neurons and astrocytes (Tsc1GFAPCKO mice). Sleep studies utilizing EEG, EMG, and behavioral analysis found that Tsc1GFAPCKO mice have decreased REM sleep and impaired sleep-wake differentiation between light and dark phases. mTOR activity and orexin expression were increased in hypothalamic sections and cultured hypothalamic neurons from Tsc1GFAPCKO mice. Both the sleep abnormalities and increased orexin expression in Tsc1GFAPCKO mice were reversed by rapamycin treatment, indicating their dependence on mTOR activation. An orexin antagonist, suvorexant, also restored normal REM levels in Tsc1GFAPCKO mice. These results identify a novel mechanistic link between mTOR and orexin in the hypothalamus related to sleep dysfunction and suggest a targeted therapeutic approach to sleep disorders in TSC.

1. Introduction

Sleep represents a critical, evolutionary-conserved physiological process required for maintenance of normal function and maturation of the central nervous system (Schwartz and Kilduff, 2015). In turn, sleep disorders may have disruptive effects on brain function and quality of life. While environmental factors strongly influence the sleep-wake cycle, genetics and other intrinsic biological mechanisms also regulate sleep homeostasis. Deeper understanding of these genetic and biological mechanisms of sleep regulation will reveal important insights into normal brain function and sleep disorders.

Tuberous sclerosis complex (TSC) is a genetic disease, causing tumor or hamartoma formation in the brain and other organs related to dysregulation of the mechanistic target of rapamycin (mTOR) pathway, a key regulator of protein synthesis, cellular growth, and other downstream biochemical and molecular mechanisms (DiMario et al., 2015; Orlova and Crino, 2010). As the tuberous sclerosis genes, TSC1 and TSC2, normally inhibit mTOR, the mTOR pathway is hyperactivated in TSC, promoting tumor growth and epileptogenesis, and mTOR inhibitors are a proven treatment for tumors and epilepsy in TSC (French et al., 2016; Krueger et al., 2010). In addition to epilepsy, intellectual disability, and autism, sleep disorders are a common neurological symptom and cause of decreased quality of life in TSC patients, including reduced total sleep time and efficiency, increased awakening, and decreased REM sleep (Bruni et al., 1995; Hunt, 1993; Hunt and Stores, 1994). The pathophysiology of sleep disorders in TSC is poorly understood and may involve a number of environmental, psychosocial, and biological factors. The existence of mouse models of other neurological manifestations of TSC afford the opportunity to investigate mechanisms of sleep dysfunction (Lipton et al., 2017), but there are very few mechanistic studies of sleep disorders in TSC. In this study, we report a novel model of sleep disorders in TSC, which allow investigations into basic mechanisms of sleep regulation involving mTOR.

Normal sleep and sleep-wake transitions depend on complex interactions between cortical and subcortical regions involved in controlling arousal (Schwartz and Kilduff, 2015). Cholinergic and monoaminergic neurons in the brainstem reticular activating system send widespread projections to the thalamus and cortical areas to maintain wakefulness. Modulation by hypothalamic nuclei and other subcortical structures regulates sleep-wake transitions. In particular, clusters of orexin-
containing neurons in the lateral hypothalamus interact extensively with the cholinergic and monoaminergic brainstem nuclei to stimulate arousal (Chow and Cao, 2016). Deficiency or dysfunction of the orexin system may lead to pathological sleep-wake states, including hypersomnia and narcolepsy (de Lecea and Huerta, 2014; Liblau et al., 2015), whereas increased orexin levels are associated with insomnia (Tang et al., 2017). In this study, we implicate a novel mechanistic link between mTOR and orexin in mediating disregulated sleep in a mouse model of TSC.

2. Materials and methods

2.1. Animals and drug treatment

Care and use of all mice were conducted according to an animal protocol approved by the Washington University School of Medicine (WUSM) Animal Studies Committee, and consistent with National Institutes of Health (NIH) guidelines on the Care and Use of Laboratory Animals. In addition, NIH guidelines on Rigor and Reproducibility in Preclinical Research were followed, including use of randomization, blinding, both sexes, and statistical/powers analyses. Both male and female mice were used and no significant differences between the sexes were detected, although the study was not designed to detect small sex differences. Tsc1fl/fl-GFAP-Cre knock-out (Tsc1fl/fl-GFAPCKO) mice with a mixed C57BL/6 and SV129 genetic background and conditional inactivation of the Tsc1 gene in both neurons and glia were generated as described previously (Uhlmann et al., 2002; Zeng et al., 2008). Although Tsc1fl/fl-GFAP-Cre mice have been shown to also involve extensive neuronal Cre-recombination (Fraser et al., 2004; Zou et al., 2017). Tsc1fl/fl-Cre and Tsc1fl/fl/littermates have previously been found to have no abnormal phenotype and were used as control animals in these experiments. For some studies, CgGt(Rosa)26Sortm6(CAG-ZsGreen1)Hze/J (Rosa-Green Cre; Jackson Laboratory) reporter mice were crossed with GFAP-Cre mice to assess for Cre-mediated recombination in orexin neurons in hypothalamus (Zou et al., 2017).

In some experiments, three-week-old Tsc1fl/fl-GFAPCKO mice were randomized to treatment with rapamycin (LC Laboratories, Woburn, MA; 3 or 6 mg/kg/day, i.p.) or vehicle for one week, with brain tissue then harvested for western blot or immunohistochemistry analysis, or followed by video-EEG/EMG monitoring. Rapamycin was initially dissolved in 100% ethanol, stored at −20 °C, and diluted in a vehicle solution containing 5% Tween 80, 5% PEG 400 (Sigma), and 4% ethanol. In other experiments, three-week old Tsc1fl/fl-GFAPCKO mice were randomized to treatment with suvorexant (Adooq Bioscience, Irvine, CA; 20 mg/kg/day, i.p.) or vehicle-treatment groups for 9 days. Suvorexant was initially dissolved in DMSO, diluted in saline, and injected at the beginning of the light cycle.

2.2. Hypothalamic neuronal culture

Primary neuronal cultures were prepared from embryonic day 15 (E15) mouse embryos as previously described with some modifications (Zhang et al., 2010). Briefly, the hypothalami were dissected individually from embryonic brains (as similarly mentioned in the western blot section), treated with 1 × trypsin and DNTase I (Sigma), and gently dissociated by trituration in Dulbecco’s modified Eagle’s medium with 5% fetal calf serum and 5% horse serum. According to the genotype results, neurons were pooled from 2 to 3 control or Tsc1fl/fl-GFAPCKO mice per culture. Dissociated cells were seeded onto poly-A-lysine/ laminin (10 μg/ml)-coated 6-well plate with Neurobasal/B27 medium (Gibco) with 5% fetal calf serum and 5% horse serum, and cultured in a humidified atmosphere (5% CO₂) at 37 °C. After 24 h of culturing, media was exchanged.

Rapamycin at a concentration of 3 ng/ml or vehicle was added to the medium of prepared neurons and incubated for 16 h. Samples were collected and homogenized separately, and Western blotting analysis was performed to measure the ratio of orexin A and β-actin, or P-S6 and S6 as described below. All experiments were conducted on cultures on DIV 3 or 4. In separate immunocytochemical studies, some cultures were fixed with 4% PFA for measurement of the purity of neuronal cell culture.

2.3. Immunohistochemistry/immunocytochemistry

Histological analysis was performed in a blinded fashion to assess orexin positive cell size and number by standard methods. In brief, brains were perfusion-fixed with 4% paraformaldehyde and cut into 45 μm sections with a freezing microtome. Immunohistochemistry was performed for orexin A to assess orexin positive neuronal cells, by labeling with primary antibody, anti-orexin A (#ab62124, Abcam, 1:200), followed by labeling with secondary antibody Alexa-488 conjugated goat anti-rabbit IgG (#A11034, Life Technologies, 1:300). In addition, sections were counterstained with TO-PRO-3 Iodide (Life Technologies, 1:1000) for the nonspecific nuclear staining of all cells. In other experiments, hypothalamic sections obtained from Rosa-Green Cre reporter mice crossed with GFAP-Cre mice were double-labeled with anti-orexin antibody to assess the percentage of orexin positive cells undergoing Cre-recombination.

Images were acquired with a Zeiss LSM PASCAL confocal microscope (Zeiss, Thornwood, NY). In images from coronal sections at approximately 2 mm posterior to bregma, orexin A positive cell numbers were counted in the regions of interest (0.445 mm²; lateral hypothalamic area) from two sections per mouse, from a total of six to eight mice per group. The size of orexin A positive cells in each group was measured by outlining the cell body and calculating area using ImageJ software (NIH, Bethesda, MD). Orexin expression was also quantitatively analyzed by measuring mean intensity within orexin neurons (minus background intensity) with ImageJ software (Jensen, 2013).

To assess the purity of neuronal cell culture, immunocytochemistry was performed. After incubating with primary antibodies (anti-NeuN mouse, MAB377, Millipore, 1:300, and anti-orexin antibody, #12389, Cell Signaling Technology, 1:500), cultures were labeled with secondary antibody Alexa-488 conjugated goat anti-mouse IgG (#A11001, Life Technologies, 1:300) and Cy3 conjugated goat anti-rabbit IgG (#A10520, Life Technologies, 1:1000), followed by labeling with secondary antibody Alexa-488 conjugated goat anti-rabbit IgG (#A11034, Life Technologies, 1:300) and Cy3 conjugated goat anti-rabbit IgG (#A10520, Life Technologies, 1:300), and finally, were counterstained with TO-PRO-3 Iodide (Life Technologies, 1:1000) for the nonspecific nuclear staining of all cells. Images of cultures (0.445 mm²) were acquired by confocal microscope and the percentage of NeuN-positive cells or GFAP-positive cells over the total TO-PRO-3-positive cells was calculated.

2.4. Western blotting

Western blot analysis was used to measure protein levels of orexin and P-S6 in the brains or hypothalamic cell cultures of Tsc1fl/fl-GFAPCKO and control mice, using standard methods as described previously (Zeng et al., 2008). Briefly, brains were dissected and placed ventral side up. Fine forceps were used to separate the hypothalamus from the preoptic area approximately 2 mm deep. The forceps were then placed caudal to the hypothalamus and used to separate the thalamus and midbrain portions (approximately 2 mm deep) and gently remove the hypothalamic tissue. The hypothalamus was then homogenized at 10 a.m. Equal amounts of total protein extract were separated by gel electrophoresis and transferred to nitrocellulose membranes. β-actin was used as a loading control. After incubating with primary antibodies to orexin A (#ab62124, Abcam, 1: 500), β-actin, P-S6 and S6 (#4970, #2215, #2217, Cell Signaling Technology, 1:10,000, 1:1000 and 1:10,000), the membranes were reacted with a peroxidase-conjugated secondary antibody (#7074, Cell Signaling Technology, 1:1000).

Signals were detected by enzyme chemiluminescence (GE Healthcare Life Science) and quantitatively analyzed with ImageJ software (NIH, Bethesda, MD). In western blot, the primary orexin band is identified at 13 kDa as the full orexin protein (orexin A preprotein).

2.5. Electroencephalography (EEG) and electromyography (EMG) electrode surgery

For sleep studies, mice received surgery for placement of EEG and EMG electrodes at 21 days of age, using methods previously described with some modifications (Anastasaki et al., 2019). Custom wire EEG/EMG electrode sets were constructed using six Teflon coated stainless steel wires (76 μm bare diameter) soldered to an electronic pin header. For EEG, a screw was attached to the opposite end of the wire and for EMG electrodes the Teflon coating was removed approximately 2 mm from the end of the wire. The soldered contacts were covered with dental cement and the electrode set sterilized for implantation.

Mice were placed under 3–4% isoflurane on a stereotaxic frame with a heating pad set to 36.5 °C until pedal withdraw reflex ceased. The skin was prepared with betadine and alcohol wipes with isoflurane maintained at 1–1.5% for the remainder of the procedure. After a midline vertical incision to expose the skull, forceps and 3% hydrogen peroxide were used to remove any connective tissue and dry the skull for electrode placement. Burr holes for the frontal reference electrode were made (anterior +0.8 mm, lateral 0.5 mm; bregma) using a micro drill with a 0.7 mm tip and screws were secured in the skull. Two bilateral “active” recording electrodes were placed over the parietal cortex (posterior −2.5 mm, lateral ± 1.5; bregma) and a ground screw secured over the cerebellum (posterior −6.4 mm, lateral ± 0.5; bregma) using the same technique as the reference electrode. The exposed end of the two wire electrodes were inserted in the neck muscle for nuchal EMG recordings with the coated portion of the wire bent to follow the contour of the head. The exposed skull and all wires were covered in a layer of dental cement (SNAP, Parkell) with the pin header secured to the head for subsequent recording. The skin was sutured around the exposed dental cement/pin header and tissue glue (Vetbond, 3 M) used to close the remainder of the incision. Mice received Buprenorphine (0.1 mg/kg, i.p.) and recovered in a warmed chamber for 2 h then recovered in recording cages.

2.6. EEG/EMG monitoring and sleep analysis

Groups of four mice (2 control; 2 Tsc1GFAPCKO) recovered from surgery in individual caging at least 72 h prior to connection with a custom flexible cable attached to the exposed pin header for recording. Freely moving tethered mice then acclimated to the recording cage for four days with normal 12-hour light/dark cycles. Bilateral cortical EEG signals were acquired using a referential montage with Stellate amplifiers and acquisition software. Signals were amplified at 10,000 × with highpass (0.5 Hz) and lowpass (100 Hz) filters applied. EMG signals were filtered with highpass (10 Hz) and lowpass (300 Hz) filters. EEG and EMG signals were digitized at 200 Hz and collected for 2 consecutive days (48 h total).

Bilateral EEG and nuchal muscle EMG files (.edf) were imported into ADInstruments LabChart software and a digital bandpass (1–35 Hz EEG; 10–100 Hz EMG) filter applied for review, as previously described (Anastasaki et al., 2019). Mice were manually scored in 10-second epochs and vigilance state scored as awake, non-REM (NREM) sleep or REM sleep using a combination of the video, EEG, EMG and respective spectral power representations. Vigilance state scoring parameters were defined based on standard criteria for adult rodents. Wakefulness was defined as periods of cyclic lower amplitude mixed frequency EEG and high tone muscle activity EMG for greater than half of the epoch duration. Brief arousal periods with 5–10 s of high muscle tone in between sleep transitions were also scored as awake. EEG periods dominated by higher amplitude delta wave activity with nuchal muscle atonia were scored as NREM sleep epochs. REM sleep consisted of periods of semi-uniform theta activity EEG with muscle atonia and/or muscle atonia with brief myoclonic twitches. Real-time power spectral analysis was simultaneously used to assess vigilance state and could also clearly differentiate awake, NREM, and REM sleep based on frequency and power distribution of the EEG and EMG data (Fig. 5B). Mice that had documented seizures on video-EEG were excluded from analysis to eliminate any effects of seizures on awake-sleep parameters.

2.7. Statistical analyses

All statistical analysis was performed using GraphPad Prism (GraphPad Software). For western blot and immunohistochemical studies, quantitative differences between groups were analyzed by one-way ANOVA with Tukey’s multiple comparisons post hoc tests. For sleep studies, differences between treatment groups and genotype were analyzed by a two-way ANOVA with repeated measures followed by Bonferroni post-tests and differences between light and dark cycle by Student’s t-test. Quantitative data are expressed as mean ± SEM. Statistical significance was defined as p < .05.

3. Results

3.1. Orexin expression and neuronal size are increased in the hypothalamus of Tsc1GFAPCKO mice in an mTORC1-dependent manner

Orexin neurons in the hypothalamus have been shown to regulate the sleep-wake cycle and been implicated in sleep disorders, such as narcolepsy. To assess orexin neurons in Tsc1GFAPCKO mice, immunohistochemistry studies were performed in hypothalamic sections from 4 week old Tsc1GFAPCKO and control mice. The cell size of orexin-expressing neurons was elevated in Tsc1GFAPCKO mice (Fig. 1A,B). Rapamycin treatment blocked this increased orexin cell size, indicating that the cytoskeletal mTOR complex 1 (mTORC1) dependent. There was a “trend” toward increased number of orexin-positive cells, but this was not statistically significant (Fig. 1C). The intensity of orexin expression in hypothalamic of Tsc1GFAPCKO mice was also quantified and found to be increased in Tsc1GFAPCKO mice compared to control mice (Fig. 1D), suggesting that total orexin protein expression was increased in Tsc1GFAPCKO mice.

To directly assess aberrant mTORC1 activity in hypothalamus, we performed western blot studies of hypothalamic homogenates derived from Tsc1GFAPCKO mice, using the ratio of P-S6 expression to total S6 expression as a measure of mTORC1 activity. The relative P-S6 expression was significantly increased in hypothalamic homogenates derived from Tsc1GFAPCKO mice compared with control mice (Fig. 2). Rapamycin treatment predictably reversed the elevated mTORC1 activity in hypothalamic neurons from Tsc1GFAPCKO mice.

We next examined the expression of orexin by western blotting of hypothalamic homogenates derived from Tsc1GFAPCKO mice. Orexin expression was significantly increased in the hypothalamus from Tsc1GFAPCKO mice compared to control mice (Fig. 3; Supplementary Fig. S1A). Rapamycin treatment blocked this increased orexin expression in Tsc1GFAPCKO mice, indicating that the orexin expression was dependent on mTORC1 activation.

As the increased orexin expression could result from an effect of Tsc1 inactivation and mTORC1 activation directly in neurons or indirectly involving astrocytes, GFAP-Cre mice were crossed with Rosa reporter mice to assess Cre recombination directly in orexin neurons of the hypothalamus. Double-labeling studies showed that ~30% of orexin-positive cells were also positive for Rosa Green, indicating a moderate efficiency of Cre recombination in orexin-expressing neurons (Supplementary Fig. S2). Thus, while an effect of astrocytes on hypothalamic function is difficult to rule out, the increase in cell size, mTORC1 activity, and orexin expression in hypothalamic neurons of Tsc1GFAPCKO mice is likely due, at least in part, to Tsc1 gene
inactivation directly in these neurons.

3.2. mTORC1 activation and orexin expression is also increased in cultured hypothalamic neurons of Tsc1GFAPCKO mice

To investigate further whether the increased mTORC1 activation and orexin expression were related to a direct effect on hypothalamic neurons, we next performed western blotting in cultured hypothalamic neurons derived from Tsc1GFAPCKO and control mice. mTORC1 activity as again measured by the ratio of P-S6 to total S6 expression was elevated in cultured hypothalamic neurons, compared with control neurons (Fig. 4). Rapamycin treatment again reversed the elevated mTORC1 activity in hypothalamic cultures from Tsc1GFAPCKO mice. Although the signal intensity was weak, orexin expression did also appear to be increased in hypothalamic neuronal cultures from Tsc1GFAPCKO mice compared to controls (Supplementary Fig. S1B,C). Rapamycin administered to hypothalamic cultures blocked the increase in orexin seen in neurons from Tsc1GFAPCKO mice. To assess the purity of our neuronal cultures, double-labeling and quantification by immunocytochemistry revealed that 89.4% ± 1.2% of cultured hypothalamic cells are NeuN-positive and 1.8% ± 0.2% are GFAP-positive, suggesting that any differences between groups was predominantly due to direct neuronal effects. Furthermore, rapamycin treatment did not affect the cell densities in culture (62.5 ± 4.7 cells/400 μm² with vehicle, 62.8 ± 3.4 cells/400 μm² with rapamycin). Overall, these findings provide additional evidence that the increased orexin and mTORC1 activation occurs directly within the orexin neurons themselves.

Fig. 1. Orexin-expressing neurons demonstrate increased cell size in hypothalamus of Tsc1GFAPCKO mice. A, Orexin-positive cells in hypothalamus were assessed by orexin A (green) immunohistochemistry, followed by counterstaining with TO-PRO-3 iodide (blue) for the nonspecific nuclear staining of all cells. B, Four-week-old Tsc1GFAPCKO mice (KO + Veh) had significantly increased orexin A positive cell size in the hypothalamus compared with control mice (Cont + Veh). Rapamycin treatment (3 mg/kg/day i.p. for one week starting at 3 weeks of age) significantly reduced the orexin A positive cell size of Tsc1GFAPCKO mice (KO + Rap) back to control levels. C, There was a trend toward increased orexin-A cell number in Tsc1GFAPCKO mice, but there was no statistically significant differences between groups in cell number. D, Mean intensity of orexin expression in hypothalamus was significantly increased in Tsc1GFAPCKO mice compared with control mice, and was reversed by rapamycin. *p < .05, **p < .01, ***p < .001 by one-way ANOVA (n = 6–8 mice/group). Cont = control mice, KO = Tsc1GFAPCKO mice, Veh = vehicle, Rap = rapamycin. Scale bars = 100 μm (low power), 50 μm (high power inset). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)
3.3 Tsc1GFAPCKO mice have sleep abnormalities, which are at least partially reversed by rapamycin and suvorexant

Given the abnormalities in orexin expression, we next investigated whether Tsc1GFAPCKO mice have any evidence of sleep abnormalities. Continuous video-EEG/EMG was performed for 48 h in four week old Tsc1GFAPCKO and control mice and the amount of time spent in each vigilance state (awake, NREM sleep, REM sleep) was assessed during both light and dark phases. Mice that had seizures were excluded from analysis. Tsc1GFAPCKO mice exhibited increased awake time in the light cycle and decreased REM sleep in both the light and dark cycles compared to control mice (Fig. 5). Furthermore, while control mice exhibited reduced wakefulness and increased NREM and REM sleep in the light phase compared to the dark, Tsc1GFAPCKO mice did not display any such sleep-wake differences between the light and dark cycles. Rapamycin treatment reversed these sleep abnormalities in Tsc1GFAPCKO mice, at least partially returning normal levels of REM sleep and fully restoring the reduced wakefulness in the light cycle and the overall differences in awake and non-REM and REM sleep times between the light and dark cycles seen in control mice (Fig. 5C–E).

4. Discussion

In this study, we have described a novel mouse model of sleep disorders in TSC, which recapitulate some of the sleep abnormalities described in TSC patients. Furthermore, we demonstrate that the sleep abnormalities are dependent on the mTOR pathway and a downstream link to increased orexin expression in hypothalamic neurons. This study provides novel insights into the mechanisms of sleep disorders in TSC and suggests innovative, targeted therapeutic strategies involving regulators of mTOR and orexin systems.
Sleep disorders are a common cause of morbidity in TSC patients, contributing to a significant decrease in quality of life for both the patients and caregivers (Bruni et al., 1995; de Vries et al., 2018; Hunt, 1993; Hunt and Stores, 1994). In one survey of 300 TSC patients, 60% reported sleep problems, primarily involving sleep initiation or maintenance (Hunt, 1993). In the more recent TOSCA database study

Fig. 5. Tsc1GFAPCKO mice have sleep abnormalities, which are reversed by rapamycin and suvorexant. A, EEG and EMG recordings, as well as video analysis, allowed determination and scoring of awake and sleep (NREM - nonREM, and REM) states using 10 sec epochs as defined in the Methods. B, A representative 20 min total power color density spectral array (CDSA) of EEG and EMG data also demonstrates clear distinction of vigilance states. C-E, Control mice (Cont + Veh) exhibit significantly decreased wakefulness and increased nonREM and REM sleep in the light phase compared with the dark. In contrast, Tsc1GFAPCKO mice (KO + Veh) did not demonstrate vigilance state differences between the light and dark phases. Tsc1GFAPCKO mice (KO + Veh) also had increased awake time in the light phase and decreased REM sleep in both light and dark phases, compared with control mice (Cont + Veh), and these differences were reversed by rapamycin (KO + Rap). F-H, Suvorexant treatment of Tsc1GFAPCKO mice (KO + Suv) reversed the decreased REM sleep in Tsc1GFAPCKO mice (KO + Veh), but had no effect on REM sleep in control mice (Cont + Suv) or on the lack of difference between the light and dark phases in Tsc1GFAPCKO mice. *p < .05, by two-way ANOVA (n = 8 mice/group); # p < .05 by t-test. Cont = control mice, KO = Tsc1GFAPCKO mice, Veh = vehicle, Rap = rapamycin, Suv = suvorexant.
involving over 2000 TSC patients, sleep difficulties were the second most common neuropsychiatric symptom, reported in 44% of patients (de Vries et al., 2018). Polysomnographic studies objectively documented sleep abnormalities in 9 of 10 TSC patients, including reduced total sleep time and efficiency, increased awakenings, and reduced REM sleep (Bruni et al., 1995). In addition to causing disruptive sleepiness and excessive daytime sleepiness, these sleep disturbances may exacerbate neurological comorbidities of TSC, such as epilepsy, cognitive dysfunction, and neuropsychiatric symptoms. In the present preclinical study, Tsc1GFAPCKO mice were found to recapitulate some aspects of sleep disorders in human TSC, including decreased REM sleep and a disrupted sleep-wake cycle (e.g., increased awake time in the light cycle when mice normally sleep more, and an overall lack of difference in awake and sleep times between the light and dark cycles).

This mouse model does not perfectly recapitulate all aspects of sleep disorders in human TSC, such as decreased total sleep time and increased sleep fragmentation, as well as the potential impact of seizures in sleep. We intentionally excluded mice that had seizures in an attempt to isolate intrinsic sleep abnormalities, but will analyze the effects of seizures and interictal spikes on the sleep-wake cycle in Tsc1GFAPCKO mice in future studies. The control mice in our experiments also had increased sleep at baseline compared to some published values in some mouse strains (Franken et al., 1999) and our previous studies in older mice (Anastasaki et al., 2019). These differences are likely related to the relatively young age of the mice and the short acclimation period, as we were attempting to complete these studies before the onset and progression of seizures which occurs at a relatively young age in these mice (Erbayat-Altay et al., 2007; Zeng et al., 2008).

The pathophysiology of neurological manifestations of TSC, such as epilepsy, may at least partially relate to focal cortical malformations, called tubers, which commonly occur in this disease. Independent of structural lesions of the brain, however, other cellular and molecular mechanisms likely promote seizures and other neurological symptoms of TSC, particularly the involvement of the mTOR pathway (Wong, 2010). mTOR is a cell signaling pathway that regulates a number of physiological processes, including cell growth, metabolism, and protein synthesis, many of which may influence epileptogenesis and other neurological manifestations of TSC. In fact, mTOR inhibitors have recently been approved for treating epilepsy in TSC patients (French et al., 2016).

Unlike epilepsy, relatively little is understood about the pathophysiology of sleep dysfunction in TSC, and treatments for sleep disorders are very limited and non-specific. Our results in a preclinical model indicate that mTOR is involved in the pathogenesis of sleep dysfunction in TSC and that mTOR inhibitors have potential therapeutic applications for sleep disorders in TSC, with rapamycin at least partially reversing the decreased REM sleep and impaired sleep-wake differences between the light and dark cycles in Tsc1GFAPCKO mice. However, the downstream mechanisms mediating mTOR effects on sleep-wake cycle are not known. Our study also demonstrates a novel interaction between mTORC1 and orexin neurons in the hypothalamus and implicates increased orexin in contributing to the sleep phenotype. As a major function of mTORC1 is to regulate protein translation, mTOR may directly influence the synthesis and expression of orexin. While GFAP is primarily localized to mature astrocytes, GFAP is also expressed in embryonic neuroprogenitor cells, thus also leading to Tsc1 inactivation in large populations of neurons, as well as astrocytes, in Tsc1GFAPCKO mice (Fraser et al., 2004; Zou et al., 2017). Our findings of increased mTORC1 activation and orexin expression in isolated hypothalamic neuronal cultures, with reversal by rapamycin, suggest that Tsc1 inactivation or mTORC1 hyperactivation occur directly within the orexin neurons themselves, leading to the increased orexin expression. Furthermore, the increased cell size, which is also prevented by rapamycin, also indicates that hyperactivation of mTORC1, a known regulator of cell size, occurs autonomously in hypothalamic neurons of Tsc1GFAPCKO mice. Finally, Cre-reporter studies indicate that Tsc1 inactivation occurs directly in at least a subset of orexin neurons. Thus, there are multiple lines of evidence suggesting that Tsc1 gene inactivation directly increases orexin expression in hypothalamic neurons. However, as Tsc1GFAPCKO mice also have well-documented effects on astrocytes (Jansen et al., 2005; Uhlmann et al., 2002; Wong et al., 2003), it is difficult to rule out the possibility that Tsc1 inactivation in glia indirectly affects hypothalamic orexin expression via astrocyte-neuronal interactions in Tsc1GFAPCKO mice, in addition to or instead of direct effects on orexin neurons. Future studies utilizing neuronal-specific conditional knockout mice will further address the cellular specificity of the increased orexin expression.

A limitation of this study is the relatively weak orexin signal in the western blotting studies, especially in culture. This could confound interpretation of differences in orexin expression between groups, but presumably this would primarily affect decreases in expression, not the reported increase in orexin expression in Tsc1GFAPCKO mice. Furthermore, considering the consistent results across both western blot (in both hypothalamic homogenates and neuronal cultures) and immunohistochemical (including both orexin intensity and neuronal cell size) studies, multiple lines of evidence collectively support the increased orexin expression in Tsc1GFAPCKO mice.

Another potential weakness of the study relates to sleep analysis in rodents, which generally has more limited technical assessment of state characteristics than human sleep studies. In particular, the use of EEG/EMG/video only may be limited in distinguishing the calm awake state and REM sleep, both of which may have reduced EMG activity and a predominant theta rhythm on EEG. However, on both routine visual inspection and on spectral analysis, calm awake usually has higher power EMG and a more mixed frequency EEG compared with REM sleep, which has a very uniform, dominant theta frequency on EEG. It’s possible that the Tsc1GFAPCKO mice later develop a dysrhythmia which may make this distinction more difficult and confound the apparent difference between control and Tsc1GFAPCKO mice, but, besides the differences in time spent in certain states, the 4 week-old Tsc1GFAPCKO mice otherwise appear to have normal state characteristics and differentiation as control mice. Nevertheless, the apparent decrease in REM sleep in the Tsc1GFAPCKO mice may also consist of a component of calm awake with low EMG.

As orexin deficiency causes hypersomnia and narcolepsy and orexin excess is associated with insomnia (de Lecea and Huerta, 2014; Fujiki et al., 2003; Liblau et al., 2015; Mieda et al., 2004; Piper et al., 2000; Tang et al., 2017), the increased orexin expression in Tsc1GFAPCKO mice represents a rational mechanism for the sleep dysfunction in these mice. As suvorexant restored the decreased REM sleep in our Tsc1GFAPCKO mice back to control levels, this indicates that orexin at least partially contributes to the sleep phenotype in these mice. However, other mechanisms downstream from mTOR independent of orexin could also be involved in part of the sleep phenotype, as suvorexant did not reverse the impaired sleep-wake differences between light and dark cycles in Tsc1GFAPCKO mice. While orexin antagonists are proven treatments for insomnia in general (Herring et al., 2012; Winrow and Renger, 2014), these results suggest that orexin antagonists may be a mechanistically-targeted treatment specifically for sleep disorders in TSC. Furthermore, independent of orexin, our results are novel and impactful in clearly implicating the mTORC1 pathway in sleep dysfunction in a TSC mouse model and supporting the potential of mTOR inhibitors as a treatment for sleep disorders in TSC.

Declaration of Competing Interest

The authors have no conflicts of interest to declare.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.nbd.2019.104615.

References


