# Face Validation and Pharmacologic Analysis of *Sik3*$^{Sleepy}$ Mutant Mouse as a Possible Model of Idiopathic Hypersomnia

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## Abstract

Idiopathic hypersomnia (IH) is a chronic neurologic disorder with unknown mechanisms that result in long night-time sleep, daytime sleepiness, long non-refreshing naps, and difficult awakening presenting as sleep drunkenness. IH patients are typically diagnosed by shorter sleep latency on multiple sleep latency test (MSLT) along with long sleep time. Only symptomatic drug treatments are currently available for IH and no animal model to study it. *Sleepy* mice carry a splicing mutation in the *Sik3* gene, leading to increased sleep time and sleep need. Here we used a mouse version of MSLT and a decay analysis of wake EEG delta power to validate the *Sleepy* mutant mouse as an animal model for IH. *Sleepy* mice had shorter sleep latency in the dark (active) phase than wild-type mice. They also showed lower decay of EEG delta density during wakefulness, possibly reflecting increased sleep inertia. These data indicate that the *Sleepy* mouse may have partial face validity as a mouse model for idiopathic hypersomnia. We then investigated the effect of orexin-A and the orexin receptor 2-selective agonist YNT-185 on the sleepiness symptoms of the *Sleepy* mouse. Intracerebroventricular orexin-A promoted wakefulness for 3 h and decreased wake EEG delta density after injection in Sleepy mice and wild-type mice. Moreover, Sleepy mice but not wild-type mice showed a sleep rebound after the orexin-A-induced wakefulness. Intraperitoneal YNT-185 promoted wakefulness for 3

hours after injection in *Sleepy* mice, indicating the potential of using orexin agonists to treat not only orexin deficiency but hypersomnolence of various etiologies.

Keywords: idiopathic hypersomnia; sleep inertia; *Sleepy* mouse; orexin; orexin agonist.

## 1. Introduction

Idiopathic hypersomnia (IH) is one of the main central disorders of hypersomnolence which is characterized by excessive daytime sleepiness, long night sleep, and long daytime naps (Sateia, 2014). Despite increased sleep time, IH patients suffer severe sleep inertia after awakening, referred to as “sleep drunkenness” (Anderson et al., 2007; Kretzschmar et al., 2016; Roth et al., 1972). According to the International Classification of Sleep Disorders, third edition (ICSD-3), IH patients are diagnosed by sleep latency time of ≤ 8 min on multiple sleep latency test (MSLT) along with long sleep time on polysomnography (Sateia, 2014). No animal model has been established to study IH (Billiard and Sonka, 2016).

Drug treatment for IH is still borrowed from narcolepsy. Options include modafinil, amphetamine, dextroamphetamine, methamphetamine, and methylphenidate (Morgenthaler et al., 2007). There was no officially approved drug for IH until August 2021, when the FDA approved a low-sodium formulation of oxybate for the treatment of idiopathic hypersomnia. However, it has the risk of central nervous system depression

and abuse and misuse ("FDA Grants First of its Kind Indication for Chronic Sleep Disorder Treatment | FDA," n.d.). In addition, its use is associated with adverse effects that have led to treatment discontinuation of 17% of the participants in a randomized withdrawal study (Dauvilliers et al., 2022). A previous study implicated an upregulated $GABA_A$ signaling in idiopathic hypersomnia pathophysiology, suggesting that the $GABA_A$ antagonist flumazenil can improve vigilance in hypersomnolent patients (Rye et al., 2012). Then, in a retrospective review, 39% of treatment-refractory hypersomnolence patients had sustained clinical benefit from sublingual and transdermal flumazenil (Trotti et al., 2016). However, another study reported absence of $GABA_A$ modulation in IH patients and discouraged the use of $GABA_A$ antagonists in IH treatment (Dauvilliers et al., 2016).

The *Sleepy* mutant mouse was discovered through a forward-genetic screening of randomly mutagenized mice. It has longer non-rapid eye movement (NREM) sleep time, especially during the active, dark phase and constitutively higher sleep need as indexed by higher NREM sleep EEG delta density. *Sleepy* phenotype results from a splicing mutation of the *Sik3* gene that leads to an in-frame deletion of 52 amino acids encoded in exon 13, encompassing the protein kinase A (PKA) phosphorylation site S551 of the SIK3

54 protein. Further experiments on invertebrates showed that *Sik3*$^{Slp}$ is a gain-of-function
55 allele (Funato et al., 2016).

56 In this study, we propose the *Sleepy* mouse as a mouse model for IH. To test the face
57 validity of this model, we used a mouse version of MSLT to assess active-phase
58 sleepiness in *Sleepy* mice. Sleep inertia is a physiological phenomenon characterized by
59 impaired cognitive and physical performances and higher tendency to go back to sleep
60 just after awakening (Tassi and Muzet, 2000). Wake EEG during sleep inertia is
61 characterized by higher delta power (Vallat et al., 2019), especially in posterior brain
62 regions (Ferrara et al., 2006; Marzano et al., 2011). Delta power is also increased during
63 wakefulness in *Sleepy* mice, which may reflect increased sleep pressure (Funato et al.,
64 2016), but the change of delta power across wake time is unknown. A previous study in
65 rodents showed that the first few minutes after awakening are associated with lower
66 neuronal firing rate and more off periods (Vyazovskiy et al., 2014). However, there are
67 no established EEG markers for sleep inertia in mice. In this study, we analyzed the decay
68 of EEG delta density after awakening in *Sleepy* and wild-type mice to investigate sleep
69 inertia.

70 Orexins are neuropeptides produced by neurons localized exclusively in the lateral
71 hypothalamus (Sakurai et al., 1998), and involved in the maintenance of wakefulness

(Chemelli et al., 1999). Loss of orexin neurons leads to narcolepsy type 1 (Thannickal et al., 2000). It is unknown whether the orexin system is impaired in *Sleepy* mouse. The nonpeptide orexin receptor 2 agonist YNT-185 promotes wakefulness in orexin-deficient narcoleptic mouse models as well as in wild-type mice without causing rebound sleep (Irukayama-Tomobe et al., 2017; Nagahara et al., 2015), indicating that it can be used to alleviate sleepiness due to causes other than orexin deficiency. Indeed, another orexin receptor 2 agonist, danavorexton, improved sleep latency in patients of not only narcolepsy type 1 but also type 2, in which the orexin level is normal (Evans et al., 2022). Therefore, we also investigated the effect of orexin-A and YNT-185 on the sleepiness symptoms of the *Sleepy* mouse to evaluate their effectiveness as a possible treatment for IH.

## 2. Materials and Methods

### *2.1. Animals*

All animal experimental procedures were conducted according to the guidelines established by the Institutional Animal Care and Use Committee of the University of Tsukuba (protocol number 22-234). *Sleepy* (*Sik3*$^{Slp/+}$) and wild-type mice used in this study were males of C57BL/6 genetic background and bred in house with in-vitro

fertilizations. *Sik3*$^{Slp/+}$ mice have a single nucleotide substitution at the splice donor site in intron 13 of the *Sik3* gene that leads to abnormal skipping of exon 13 (Funato et al., 2016). Food and water were provided ad libitum. Room temperature and humidity were maintained at 23 ± 2 °C and 55 ± 5%, respectively. All mice were housed individually and kept under 12-h dark:12-h light cycle.

### *2.2. Surgery*

The surgeries were done as described before (Miyoshi et al., 2019; Suzuki-Abe et al., 2022). isoflurane was used for anesthesia (4% for induction, 2% for maintenance). For intracerebroventricular injection, a guide cannula was implanted in the left lateral ventricle. Two stainless steel screws serving as EEG electrodes were implanted epidurally over the right frontal and parietal cortices. For EMG recording, two flexible wires were attached to the right and left neck extensor muscles. Mice were left to recover from surgery for 1 week. Then, they were tethered to a counterbalanced arm that allows for free movement. EEG and EMG recording started after 1-week acclimation to the tether.

### *2.3. Sleep behavior analysis*

Methods for sleep recording and staging were described previously (Miyoshi et al., 2019). Basal recording started from the onset of the light phase and lasted for 2 consecutive days.

For MSLT, orexin-A injection, and YNT-185 injection experiments, sleep was staged by 20-sec epochs first by a semi-automated, MatLab (MathWorks) program and then by visual inspection. For wake EEG delta power analysis, sleep was staged by 4-sec epochs, and EEG delta density during wake was calculated as the ratio of delta power (1-4 Hz) to the total EEG power (1-30 Hz) at each 4-sec epoch. We used RStudio 2021.09.0 to automatically choose Wake episodes that are longer than 5 min and preceded by 15-minute sleep episodes that are composed of more than 60% NREM sleep. Mice were 9-12 weeks old.

### *2.4. MSLT*

Different mice versions of MSLT were established to comprehensively measure sleep propensity in mice after intervention (Suzuki et al., 2013; Veasey et al., 2004). In this study we performed mouse MSLT as described previously (McKenna et al., 2007). Mice's sleep was disturbed by gentle handling for 5 min followed by 25-min sleep opportunity. These 30 mins were repeated for 6 times (Fig. 1a). The sleep latency was calculated starting from the end of gentle handling to the first appearance of 2 successive NREM-sleep epochs. Since at least 50% (10 sec) of an epoch has to be judged as NREM sleep in order for the epoch to be staged as NREM, this means at least 20 sec of NREM sleep within a 40-sec interval. Light-phase MSLT was performed at

ZT6 and dark-phase MSLT was performed at ZT14 using littermate wild-type and *Sleepy* mice aged 10-59 weeks (mean ± SEM: light phase – wild type 24 ± 5, Sleepy 24 ± 3; dark phase – wild type 24 ± 6, *Sleepy* 26 ± 5). Our preliminary data show that *Sleepy* and wild-type mice exhibit only a small difference in anxiety levels (*Sleepy* being slightly more anxious), which should not affect the MSLT results.

### *2.5. Drug administrations*

Orexin-A (Peptide Institute, Catalog No: 4346-s) was injected at ZT0 intracerebroventricularly at 2 nmol/mouse in 2 µl to the left lateral ventricle with a micro-syringe at a rate of 1ul/min. To acclimate mice for the injection, gentle restraint similar to that applied during intracerebroventricular injection was applied for 5 min for at least 3 times before the negative control and orexin injection. Artificial CSF was used as a negative control. Wild-type and *Sleepy* mice used for orexin-A injection were 23-41 weeks old (mean ± SEM: wild type 34 ± 3, Sleepy 27 ± 1). YNT-185 (synthesized by co-author T.S.) (Irukayama-Tomobe et al., 2017; Nagahara et al., 2015) was injected intraperitoneally at 40 mg/kg in 100 µl to *Sleepy* mice aged 11-17 weeks at ZT6, and saline acidified to pH 2.3 with HCl, same pH as the drug solution, was used as a negative control. To acclimate mice to the acidity of the YNT-185.2HCL solution, the acidified saline was injected for at least 3 times every other day before the actual negative control and drug injection.

### *2.6. Statistical analysis*

All statistical tests were conducted by GraphPad Prism 9.4.1 and RStudio 2021.09.0 for Windows. For data represented by boxplots in Figures, Wilcoxon rank sum test was used for two-group comparisons and Wilcoxon signed rank test was used to compare paired groups. Two-way repeated-measures ANOVA followed by Bonferroni test were used for time-course data. Difference was considered significant when $p < 0.05$.

## 3. Results

### 3.1. *Sleepy* mice have shorter sleep latency than wild-type mice in the active phase

MSLT is used in human patients to evaluate daytime sleepiness and to diagnose IH along with polysomnography (Sateia, 2014). In the standard test, the patient takes 4 nap opportunities at intervals of 2 h. The nap opportunity is ended after 20 min if there was no sleep or after 3 epochs of stage 1 sleep (Carskadon et al., 1987). As mice have much shorter sleep and wake episodes, we performed a mouse version of MSLT that is composed of 6 sleep opportunities, each lasting for only 25 min and preceded by 5 min of sleep disturbance (McKenna et al., 2007 and Fig. 1a). We performed the test in the light phase at ZT6 and in the dark phase at ZT14, since the smallest and the biggest sleep

159 time difference between wild-type and *Sleepy* mice, respectively, is observed at these
160 points of time (Funato et al., 2016). In the light phase, there was no significant difference
161 of the sleep latency after each trial or the average sleep latency across the first 3 trials
162 between *Sleepy* and wild-type mice (Fig. 1b, c). In the dark phase, wild-type mice had
163 initially longer sleep latency which dropped in the later trials, while *Sleepy* mice showed
164 constantly short sleep latency time across the six trials. Sleep latency difference between
165 wild-type and *Sleepy* mice was significant for the first trial (Fig. 1d) and for the sleep
166 latency average across the first 3 trials (Fig. 1e).

167 **3.2. *Sleepy* mice have reduced decay of EEG delta density during wakefulness**

168 Severe sleep inertia, i.e. sleep drunkenness, is a strong indicator of IH that can
169 discriminate it from other disorders of hypersomnolence (Dauvilliers et al., 2019;
170 Kretzschmar et al., 2016). In healthy humans, sleep inertia EEG is characterized by higher
171 delta power (Ferrara et al., 2006; Marzano et al., 2011; Vallat et al., 2019). Since *Sleepy*
172 mice show increased EEG delta power during wakefulness (Funato et al., 2016), we
173 investigated the decay of EEG delta density after awakening to assess sleep inertia. In this
174 analysis we included wake episodes that are longer than 5 min and preceded by
175 consolidated sleep episodes (*2.3. Sleep behavior analysis* and Fig. 2a). As expected
176 (Funato et al., 2016), *Sleepy* mice had higher average wake EEG delta density than wild-

177 type mice (fig. 2b). We found that EEG delta density decreased gradually after awakening
178 in both wild-type and *Sleepy* mice to reach wake delta average after about 5 min (Fig. 2c),
179 indicating that the increased delta power during NREM sleep does not dissipate
180 immediately after waking, rather, it decreases gradually over time which can mark a
181 period of sleep inertia. Then, we compared the wake EEG delta density over the first and
182 last 2 min of each wake episode. Wake EEG delta density was higher in the first 2 min
183 than in the last 2 min in wild-type mice only (Fig 2d), suggesting that *Sleepy* mice have
184 lower decay of delta density which may reflect sleep drunkenness.

185 **3.3. Orexin-A increases wake time in *Sleepy* mice**

186 First, to confirm that the wake-inducing orexin pathway is intact in *Sleepy* mice, Orexin-
187 A was administered at 2 nmol per mouse to wild-type and *Sleepy* mice by
188 intracerebroventricular injection at ZT0. Both, wild-type and *Sleepy* mice, showed
189 marked wakefulness after injection while *Sleepy* mice but not wild-type mice showed
190 mild sleep rebound after the end of the orexin-induced wakefulness (Fig. 3a-d). The
191 increase in wake time was significant for 3 h in wild-type and *Sleepy* mice (Fig. 3g).
192 However, the increase in wake time was significantly greater in wild-type mice than in
193 *Sleepy* mice (fig. 3h). In addition, Orexin-A reduced wake EEG delta density in wild-type
194 and *Sleepy* mice after injection (Fig. 3e, f); however, the decrease in the average wake

EEG delta density was more marked in wild-type mice (Fig. 3i). These results confirm that the orexin pathway can still exert its waking effects in the presence of the *Sik3* mutation in *Sleepy* mice, although the inherently increased sleep need of *Sleepy* mice may have blunted the orexin action.

### 3.4. YNT-185 increases wake time in *Sleepy* mice

Nonpeptide OX2R agonist, YNT-185, was developed as a proof-of-concept for treating the core defect of narcolepsy type 1 (Irukayama-Tomobe et al., 2017; Nagahara et al., 2015). YNT-185 promotes wakefulness in both wild-type and narcoleptic mice without causing rebound sleep (Irukayama-Tomobe et al., 2017), indicating that it can be used to treat sleepiness due to other conditions. To investigate the effect of wake-promoting orexin agonists on the sleepiness symptoms of the *Sleepy* mice, 40 mg/kg YNT-185 was injected intraperitoneally at ZT6 to *Sleepy* mice (Fig. 4a). After injection, YNT-185 increased wake time significantly for 3 h after injection (Fig. 4b). On hourly analysis, the increase in wakefulness was significant only for the 1$^{st}$ hour after injection (Fig. 4c).

## 4. Discussion

This study showed that *Sleepy* mice have shorter sleep latency on the mouse-version of MSLT during the dark phase, the active phase for mice, indicating that *Sleepy* mice can

represent the daytime sleepiness suffered by IH patients. In addition, *Sleepy* mice showed lower decay of EEG delta density during wakefulness which may represent sleep drunkenness. Taken together with the increased total NREM sleep time in *Sleepy* mice, these data suggest partial face validity of *Sleepy* mutant mouse as a mouse model for IH. We also showed that both orexin-A and the orexin agonist YNT-185 induced wakefulness for 3 h in *Sleepy* mice, suggesting that orexin agonists could be effective to treat IH patients.

As far as we are aware, this is the first study that introduces an animal model for IH (Billiard and Sonka, 2016), which enables us to test new drugs and investigate their effects on the major complaints of IH patients, i.e. long sleep time, daytime sleepiness and sleep drunkenness, using objective parameters such as sleep time, sleep latency on the mouse version of MSLT, and the decay of wake EEG delta density. Although FDA has approved a low-sodium version of oxybate for the treatment of IH ("FDA Grants First of its Kind Indication for Chronic Sleep Disorder Treatment | FDA," n.d.), its mechanism of action is still unclear. It is thought that a night dose of sodium oxybate ameliorates nighttime sleep fragmentation and enhances sleep quality in narcolepsy patients through its action on $GABA_B$ receptors, thus alleviating daytime sleepiness and cataplexy. However, its mechanism of action in IH is not fully understood (Dauvilliers et al., 2022). In addition,

its use may be limited by serious adverse effects (Dauvilliers et al., 2022; “FDA Grants First of its Kind Indication for Chronic Sleep Disorder Treatment | FDA,” n.d.). Notably, previous studies in mice showed that sodium oxybate had no appreciable effect on sleep time either in wild-type mice or narcoleptic mouse models, while increased NREM sleep EEG delta power only for a short period after injection (Black et al., 2014; Meerlo et al., 2004). Here we tried YNT-185, the OX2R agonist originally designed to treat the core defect of narcolepsy type 1 (Irukayama-Tomobe et al., 2017). Interestingly, although the *Sleepy* mouse has a similar or even stronger response to caffeine and modafinil (Funato et al., 2016), it had a weaker response to orexin than wild-type mice. The effect of YNT-185 was also restricted to the first hour after injection. This may be because of the low efficacy and/or short pharmacokinetics of YNT-185 (Irukayama-Tomobe et al., 2017). Orexin agonism is a promising concept for the symptomatic treatment of IH patients, although a better agonist than YNT-185 would be needed.

IH mouse model can also help to understand the physiology of sleep homeostasis and the pathophysiology of IH. Sleep pressure normally increases gradually during wakefulness and dissipates during sleep (Borbely, 1982). However, what the substrates of sleep need are and how they are building up in the brain is poorly understood. The *Sleepy* mouse, as a model of inherently increased sleep need, can give insights into the

molecular substrates of sleep need in the brain. Indeed, a study compared *Sleepy* mice to sleep-deprived wild-type mice using phosphoproteomic analysis found that the phosphorylation of synaptic sleep-need-index phosphoproteins (SNIPPs) increases during wake and dissipates during sleep (Wang et al., 2018) suggesting that it is a regulatory mechanism of sleep homeostasis. Recent studies utilizing *Sleepy* mice further discovered the LKB1-SIK3-HDAC4 pathway that regulates sleep depth and sleep amount in the excitatory neurons of the cerebral cortex and the hypothalamus, respectively (Kim et al., 2022; Zhou et al., 2022).

The *sleepy* mouse can give us insights on the development of IH symptoms. It shows lower level of arousal as marked by shorter sleep latency and higher sleep need as marked by higher NREM sleep EEG delta density (Funato et al., 2016), which are independently regulated (Suzuki et al., 2013). It also represents severe sleep inertia and longer sleep time (Funato et al., 2016) manifested in IH patients (Evangelista et al., 2021; Vernet and Arnulf, 2009); sleep inertia is associated with a higher score on the IH severity scale (IHSS) in patients (Dauvilliers et al., 2019). However, it is still unclear if higher wake EEG delta density in mice is associated with impaired performance as expected in sleep inertia; assessing performance in mice during the limited time period immediately after waking up is technically challenging.

As the pathophysiology and the genetic background of IH is still unclear, one limitation of this study is that the *Sleepy* mouse is an isomorphic model of IH, i.e., it mimics only the symptoms of the disorder without reflecting the pathophysiologic mechanism. *Sleepy* mouse results from a semidominant genetic mutation, while the genetic etiology of IH is still unclear with no reports of Sik3 mutation thus far, even though a genetic cause is suggested by the strong family history (Billiard and Sonka, 2016). Although a recent study demonstrated the association of a rare cleavage-site variant of the prepro-orexin gene with IH, it explained the pathophysiology in a very small fraction (1-2%) of IH patients (Miyagawa et al., 2022). Another difference between *Sleepy* mice and IH patients is that IH patients may have disrupted circadian rhythm as suggested by dampened expression of the rhythmically expressed genes BMAL1, PER1, and PER2 (Lippert et al., 2014) and prolonged circadian length (Materna et al., 2018) in these patients compared with healthy controls, while *Sleepy* mice show normal circadian length in constant darkness (Funato et al., 2016).

In addition, NREM sleep ratio and EEG delta power are higher in *Sleepy* mice (Funato et al., 2016), indicating increased homeostatic sleep pressure, while previous studies reported a decrease of NREM sleep percentage (Plante, 2018) and delta power (Sforza et al., 2000) in IH patients, suggesting that IH patients may need more sleep because of the

lower intensity of NREM sleep. That was opposed by a recent study that reported a higher percentage of NREM sleep in patients with hypersomnia (Evangelista et al., 2021). At any rate, IH lacks definitive EEG markers thus far, owing to the heterogeneity of study protocols and small sample numbers (Rugama et al., 2020).

In summary, this paper proposes the *Sleepy* mouse as a partially face-valid mouse model for IH on the basis of increased NREM sleep amounts, shorter sleep latency in the active phase, and lower decay of EEG delta density after awakening. It also shows that orexin agonists can ameliorate sleepiness of various origins. More research is required in the future to evaluate the different hypotheses suggested to explain the pathophysiology and to define EEG markers of IH.

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