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Mutant Neuropeptide S receptor reduces sleep duration with preserved memory consolidation

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One-sentence summaries: A human Neuropeptide S Receptor 1 (NPSR1) mutation found in natural short sleepers renders mutant mice to be short sleepers with more resilience to memory deficits caused by sleep deprivation.

Abstract: Sleep is a crucial physiological process for our survival and cognitive performance, yet the factors controlling human sleep regulation remain poorly understood. Here we identified a missense mutation in a G-protein coupled Neuropeptide S Receptor 1 (NPSR1) that is associated with a natural short sleep phenotype in humans. Mice carrying the homologous mutation exhibit less sleep time despite increased sleep pressure. They are also resistant to contextual memory deficits associated with sleep deprivation. *In vivo*, the mutant receptors are more sensitive to Neuropeptide S ligand treatment. These results highlight an important role for the NPS/NPSR1 pathway in human sleep duration regulation and in the connection between sleep homeostasis and memory consolidation.

Introduction

Sleep remains a relatively understudied phenomenon, despite being essential in some form to most vertebrate life. Although humans spend approximately one-third of their lives in the sleep state, an understanding and recognition of its importance for our wellbeing is severely lacking. Sleep of sufficient duration, continuity, and depth is necessary to maintain high cognitive performance during wake and to prevent certain physiological changes that may predispose individuals to many adverse health outcomes *(1–6)*. On average, people require about 8–8½ hours of sleep each day to function optimally*(7)*. However, current surveys indicate that 35–40% of the adult US population sleeps less than 7 hours on weekday nights*(8)*, a duration known to lead to cumulative deficits in behavioral alertness and vigilant attention *(9)*.

Sleep duration varies greatly (and appear to be normally distributed in the general population) among individuals and are heavily influenced by both genetic and environmental factors *(10–15)*, making their investigation especially challenging. The specific mechanisms underlying these differences are largely unknown, and until the recent identification of the first human gene/mutation linked to a short sleep duration trait, there was no knowledge regarding genetic contributions to short sleep in humans *(16)*. People with this trait (familial natural short sleep—FNSS) have a life-long tendency to sleep only 4-6 hours/night while still feeling well rested *(16)*. Anecdotally, these individuals also do not seem to

bear the greater load of comorbid disorders traditionally associated with chronically restricted sleep. Identification of human FNSS genes presents an opportunity to study not only the genetics of human sleep dynamics, but also the relationship between sleep homeostasis and health.

In this study, we identified another FNSS family and report a mutation in the *NPSR1* gene causing a short sleep phenotype. NPSR1 is a GPCR whose cognate ligand, Neuropeptide S (NPS), has been reported to modulate arousal and sleep behaviors (20). Administration of NPS in mice increases wakefulness and hyperactivity. We recreated the putative FNSS mutation- *NPSR1-Y206H-* in mice and found that those carrying the homologous mutation showed a short sleep phenotype similar to human FNSS. Further, they appeared to be resistant to certain memory deficits associated with sleep deprivation. Correspondingly, we showed the *NPSR1-Y206H* substitution changes downstream signaling dynamics and neuron behaviors in the mouse brain in response to NPS treatment. These data suggest a causative role for the *NPSR1-Y206H* mutation in the short sleep phenotype and advances our understanding of the genetic players in human sleep variability with potential therapeutic implications.

Results

NPSR1-Y206H was found in one FNSS family

In one of our identified FNSS families (K50226), the habitual total sleep time of two

subjects was much shorter (5.5 and 4.3 hours) than the average optimal sleep duration in the general population (8-8 ½ hours) *(17)* (Fig. 1A). To pinpoint potential genetic drivers, we performed whole exome sequencing of DNA samples from these two individuals and identified a shared point mutation in the Neuropeptide S Receptor 1 (*NPSR1*) gene. This mutation converts a tyrosine into a histidine at position 206 (Y206H) in both of the two known isoforms (*NPSR1A* and *B*). The mutated residue is located in one of the highly conserved extracellular domains of NPSR1 (Fig. 1, B and C). *NPSR1-Y206H i*s a rare mutation not found in the Exome Aggregation Consortium database and with a frequency of 4.06×10^{-6} in the Genome Aggregation database. NPS has been previously reported to promote arousal in rodents *(18)*. Plus, a homozygous *NPSR1-N1*07I polymorphism (Fig. 1C) was reported to be associated with slightly reduced (~20 minutes) sleep duration in the human population by genetic association studies *(19, 20)*□which all support the possibility that this mutation may be causal for the FNSS trait in affected individuals.

Npsr1-Y206H **mice show increased mobile time**

Given the highly conserved protein sequence between mouse and human NPSR1 (fig. S1A), we generated a *Npsr1-Y206H* knock-in mouse model using CRISPR/Cas9. Endogenous *Npsr1* mRNA expressions were comparable between wild type (WT, *Npsr1*+/+) and mutant (*Npsr1*+/m) mice (fig. S1B). We first monitored the activity

of WT and *Npsr1-Y206H* mice using infrared video recording (ANY-maze) *(21)*. *Npsr1-Y206H* mice demonstrated more mobile time and greater traveled distance than WT during both light and dark phases (Fig. 2A and fig. S2A). The mutant mice displayed significantly reduced mobile episodes (P = 0.0005; fig. S2B), suggesting they had more consolidated mobile periods.

Npsr1-Y206H **mice spend less time sleeping**

In order to study sleep architecture, we next monitored WT and *Npsr1-Y206H* mice with electroencephalogram/electromyogram (EEG/EMG) recordings. Consistent with the ANY-maze results, *Npsr1-Y206H* mice exhibited a reduced total sleep time of 71 minutes compared to WT (P <0.0001; Fig. 2B and fig. S2C). This difference remained statistically significant when assessed individually in both the light (P =0.003) and dark phases (P =0.0018; Fig. 2B). EEG data indicated that a reduction in non-rapid eye movement (NREM) sleep in mutants was the primary contributor to the short-sleep phenotype in both the light and dark phases, with the most pronounced change in the dark phase (Fig. 2, C and E). *Npsr1-Y206H* mice also showed significantly reduced rapid eye movement (REM) sleep during the dark phase (P =0.0084; Fig. 2, D and E). We also examined the sleep phenotype of *Npsr1* knockout (KO, *Npsr1*-/-) mice. No significant difference in mobile time or sleep duration was detected in WT vs. KO littermates (fig. S2, D and E). Taken together, these results suggest that the *NPSR1-Y206H* mutation is likely

the genetic cause of natural short sleep behavior in both mouse and human mutation carriers, probably through a gain-of-function.

Consistent with the reduced mobile episodes in the ANY-maze assay, *Npsr1-Y206H* mice showed a significant reduction in wake bouts during the dark period ($P = 0.0056$) and an increase of episode duration during both the light ($P = 0.0028$) and dark phases ($P = 0.0132$) (fig. S3, A and B). *Npsr1-Y206H* mice also showed fewer episodes of NREM/REM sleep than WT (fig. S3, C and E), while the mean duration of each NREM/REM episode was unchanged (fig. S3, D and F). Together, these results suggest that the *NPSR1-Y206H* mutation contributes to shorter sleep duration through a combination of longer average wake-bout length with higher wake-bout number and reduced number of REM/NREM bouts with unchanged bout length.

***Npsr1-Y206H* mice sustain higher sleep pressure**

Sleep pressure in mammals increases concomitantly with wake time. Spectral analysis of EEG showed an increase in the delta-range (1-4Hz) power during NREM sleep in *Npsr1-Y206H* mice (fig. S4A), a feature classically associated with increased sleep need. We next compared EEG delta power during NREM sleep across light-dark cycle between mutant and control mice. During the dark phase, *Npsr1-Y206H* mice accumulated higher delta power than WT, possibly the result of increased wake time, which dissipates during the light phase (Fig. 2F), This result

indicates that *Npsr1-Y206H* mice, though having reduced sleep time, can sustain higher sleep pressure.

Recently, quantitative phosphoproteomic analysis was performed in sleep-deprived wild type mice and the *Sleepy* mutant mouse models. High sleep pressure was found to be associated with induction of cumulative phosphorylation of the brain proteome that dissipated during sleep *(22)*. We measured expression of these proteins, called sleep-need-index-phosphoproteins (SNIPPs), in mutant and WT *NPSR1* mice as a correlate metric for sleep pressure. Phosphorylation of the SNIPPs EF2 and Synapsin-1 was consistently increased in *Npsr1-Y206H* mice at ZT2 and ZT22 but not at ZT11 when sleep pressure is lowest (fig. S4, B and C). This molecular data is consistent with the *Npsr1-Y206H* mutant animals exhibiting increased sleep pressure in the early light phase and late dark phase (Fig. 2F).

***Npsr1-Y206H* mice have normal recovery sleep after sleep deprivation**

To further characterize these mice, we examined their sleep features under high sleep pressure by subjecting them to 6-hour sleep deprivation (SD, ZT0-6). As expected, *Npsr1-Y206H* mice displayed greater delta power than WT mice in the first hour after SD (Fig. 3A). Phosphorylation of EF2 and Synapsin-1 was also consistently increased in *Npsr1-Y206H* mice following SD (fig. S5, A and B). Similar to what was observed without SD, *Npsr1-Y206H* mice displayed higher delta power

than WT mice during the early light phase and late dark phase. In addition, *Npsr1-Y206H* mice exhibited significantly reduced sleep (P = 0.0013) (REM (P = 0.0028) and NREM (P = 0.0016)) time accompanied with increased sleep pressure in the dark period immediately following SD (Fig. 3, B to D). Notably, the mutant mice showed comparable sleep gain with WT after SD (Fig. 3E), indicating that *Npsr1-Y206H* mice have a sleep rebound process that is similar to WT. Collectively, our results suggest that *NPSR1-Y206H* mutation promotes wakefulness in the presence of high sleep pressure.

Npsr1-Y206H **mice show increased phospho-CREB in the brain**

NPSR1 is a receptor coupled to both Gs and Gq signaling and widely expressed in mammalian brain *(23, 24)*. In order to determine whether the Y206H mutation alters the physiological function of NPSR1, we used brain lysates to quantify phospho-CREB− a common downstream effector of both the Gs and Gq pathways *(25)*. Increased phospho-CREB was observed in the cortex of *Npsr1-Y206H* mice at ZT2 and ZT22 (Fig. 4, A and B). To validate the putative activity of NPS on phospho-CREB, we then performed intracerebroventricular (ICV) injection of NPS. Different concentrations of NPS were injected at ZT11, when phospho-CREB is comparable between WT and mutant mice. A dose-dependent increase of phospho-CREB was observed in WT mice after NPS injection, which was completely blocked by NPSR1 antagonist SHA 68 (Fig. 4, C to E) *(26, 27)*. The increase of phospho-

CREB was further enhanced in *Npsr1-Y206H* mice (Fig. 4, C and D). No effect was observed in *Npsr1* KO mice (Fig. 4F), confirming NPS signaling is specific to NPSR1. These results revealed that NPS induces phospho-CREB in mouse cortex which was augmented in mutant mice, suggesting that mutant protein is likely to be more active *in vivo*.

Neurons from *Npsr1-Y206H* mice are hypersensitive to NPS

Since NPS/NPSR1 signaling was reported to trigger calcium mobilization in neurons *(28)*, we performed single cell calcium imaging on acutely isolated brain slices prepared from WT and mutant mice (Fig. 5A). Neurons in centromedial thalamus (CMT) have been found to induce NREM-wake transitions *(29)* and *Npsr1* mRNA is highly expressed in this area *(24)*. We thus analyzed calcium signaling in this region (Fig. 5B), categorizing NPS responsive cells into four distinct groups based on their GCaMP signal response pattern after NPS treatment (Fig. 5C). The proportion of cells in all groups was lower in brain slices from *Npsr1* KO mice (Fig. 5D), indicating that the GCaMP signals monitored here were primarily mediated by the NPS/NPSR1 pathway. In the mutant brain slices, there was a significantly higher ratio of cells with a group 2 (fast and long-lasting) type activation response to NPS (Fig. 5D). We also compared the calcium response of the neurons in lateral hypothalamus (LH) which is another well-defined sleep regulating center in the brain. Notably, there is no significant difference between WT and KO slices in all

the groups, suggesting NPS at this dose is not sufficient to initiate the NPSR1 dependent calcium response in the WT cells (fig. S6). This is probably due to the relatively lower expression of NPSR1 in this region compared with that of CMT *(24)*. Nonetheless, the percentage of cells in group 2 is significantly higher in the mutant slices suggesting the mutant cells have lower threshold for NPS response (fig. S6). Together, these results further support the conclusion that mutant receptors are more active *in vivo*.

Contextual memory of *Npsr1-Y206H* mice are more resilient to sleep loss

Accumulated sleep pressure caused by prolonged wakefulness can impair cognitive function *(6, 30, 31)*. However, the cognitive performance of human FNSS subjects seemed unimpaired despite long-term reduced sleep duration *(32)*. To see whether this phenomenon can be replicated in the *Npsr1-Y206H* mice, we subjected them to the contextual fear conditioning test, a memory-based assay known to be sensitive to sleep loss *(33, 34)*. WT and *Npsr1-Y206H* mice were trained during either the early light phase (ZT3-4) (Fig. 6A) or late dark phase (ZT23-24) (Fig. 6B) followed by testing at 24 and 48 hours after training. These time windows were chosen because sleep deprivation needs to be carried out during sleep (light) phase and the changes of phospho-CREB and sleep pressure were most prominent at these two time windows. *Npsr1-Y206H* mice had similar performance as WT on both day 1 and 2 despite having less overall sleep time and

a higher sleep pressure (Fig. 6 and Fig. 2, B and F). We subjected another group of mice to 6-hour SD immediately after training (Fig. 6, A and B), which has been shown to impair memory consolidation *(33, 34)*. WT mice showed significantly reduced freezing time (P = 0.0179, ZT3; P = 0.0488, ZT23) on day 1 indicating impaired memory consolidation. Interestingly, *Npsr1-Y206H* mice exhibited no loss of freezing time upon testing on day 1 after sleep deprivation, suggesting that contextual memory of mutant mice is more resistant to sleep loss. Notably, the mutant mice exhibited similar freezing times with WT mice on day 2, implying a preserved extinction process of contextual memory.

Discussion

The physiological function of NPS/NPSR1 pathway was first deciphered in 2004 *(18)*. Although NPS/NPSR1 signaling was shown to have strong wake-promoting effects, most of the findings were derived from studies of rodents and limited data is available about its effect on human sleep regulation. Initially found to be linked to an increased susceptibility for asthma *(35)*, the homozygous *NPSR1-N1*07I polymorphism in the human population was also reported to be associated with slightly reduced (~20 minutes) sleep duration by genetic association studies *(19, 20)* although there was no effort made to understand if/how this polymorphism was causative of the association (vs. genetically linked to it). Similar to *NPSR1-Y206H*, the *NPSR1-N107I* is also a hyperactive form (gain-of-function) in cultured

cells *(23)*. Interestingly, both Y206 and N107 residues are located at the extracellular domains (Fig.1C), implying that these mutations may increase ligand affinity or agonist efficacy rather than produce more constitutively active forms of receptor. Y206H differs from the N107I polymorphism in several ways. First, the incidence of Y206H ($4.06x10^{-6}$) is much lower than that of N107I (~0.45, http://exac.broadinstitute.org) in the population. Second, heterozygous Y206H human carriers (>2 hours shorter sleep) have much stronger sleep phenotype than homozygous N107I (~20 minutes shorter sleep) carriers (heterozygous N107I human carriers do no show sleep duration difference). Third, the Y206 residue of NPSR1 is well conserved in vertebrates, while the corresponding 107 residues in most vertebrates including mouse is Ile (fig. S1A). All these features of the Y206H mutation support its causative role for FNSS and thus strengthen the important role of NPS/NPSR1 in modulating sleep duration. Indeed, *Npsr1-Y206H* mice displayed more wakefulness in the presence of higher sleep pressure. Considering the wake-promoting function of NPS/NPSR1 *(20),* and our finding that *Npsr1-Y206H* is a gain-of-function mutation, we propose that the short sleep phenotype of human FNSS or *Npsr1-Y206H* mice results from strong induction of arousal. This hypothesis is consistent with the highly energetic behavioral traits observed in human FNSS.

Npsr1 is widely expressed in the brain, and our results indicate hypersensitivity of mutant NPSR1 to NPS in at least two separate regions that were tested.

Together, these results suggest that the wake-promoting phenotype in *Npsr1-Y206H* mice is likely mediated by the hyperactivity of mutant NPSR1 in multiple sleep-wake-regulating nuclei including CMT and LH. Interestingly, high expressions of both *Npsr1* and NPS were detected in Paraventricular thalamus (PVT) which was recently proven to be critical for wakefulness*(36)*. Moreover, moderate expression of *Npsr1* was found in some nuclei of basal forebrain regions, another known sleep regulatory area. Further in-depth investigation is needed to determine all the loci participating in NPS/NPSR sleep regulatory pathway.

Chronic partial sleep deprivation or restricted sleep often results in severe behavioral, physiological, psychiatric and cognitive disorders, including fatigue, lapses in behavioral alertness, increased risk of obesity and diabetes, adverse cardiometabolic outcomes, depression, and deficits in cognitive performance *(1, 37, 38)*. Studies of healthy adults showed that restriction of sleep time to 4 or 6 hours per night over 14 consecutive days would result in significant cumulative, dose-dependent deficits in performance on all cognitive tasks *(30)*. The humans with *NPSR1-Y206H* and mutant mouse model have a lifelong reduction in daily sleep time (resembling restricted sleep for regular sleepers) accompanied with higher sleep pressure demonstrated in mice. However, this type of chronic sleep deprivation seems to be ‘benign’ and has no obvious detrimental effects to these FNSS subjects based on our assessments of the human subjects and the learning and memory assays done in the mice. Interestingly, in our growing clinical

database of FNSS, we have observed that FNSS subjects are healthy, energetic, optimistic, with high pain threshold, and do not seem to suffer from adverse effects of chronic restricted sleep (Fig S7, questionnaires). It is possible that there is a mechanism in these FNSS human and mice compensating for the negative effects normally caused by sleep loss. Alternatively, these FNSS humans and mice are impervious to the negative effects caused by sleep loss. Further studies are needed to distinguish these possibilities. Intriguingly, NPS has been reported to facilitate learning and memory *(39–41)* and elicit anxiolytic *(18)* and antinociceptive effects *(42, 43)*. It is possible that *NPSR1-Y206H* receptor is also responsible for at least some of the seemingly protective traits (in addition to short sleep behavior) for these human mutation carriers. Whether these protective traits are secondary to short and efficient sleep or independently regulated by NPS/NPSR1 signals warrant further investigation.

Although a wake-promoting function of NPS/NPSR1 has been demonstrated by central administration of NPS *(20)*, studies from knockout mice were inconclusive as the knockout mice have a minimal (or controversial) sleep phenotype under baseline conditions depending on the mouse background *(44–47)*. The lack of a sleep phenotype in knockout studies does not necessarily undermine the importance of NPSR1 in sleep regulation. It is not uncommon that knockout mutations give no obvious phenotype, whereas gain-of-function dominant mutants provide critical mechanistic insight. Identification of the *NPSR1-Y206H* mutation in

FNSS subjects presents an opportunity to reveal the mechanism of sleep-regulation function by NPSR1.

The main limitation of this study was the difference between humans and mouse sleep patterns. In spite of the highly conserved genomic sequences, humans and mice display different features in sleep behaviors. Humans spend most sleep time at night and almost no sleep time during the day, while mice sleep both in the light and dark phases, with about 70% sleep time in the light phase and 30% in the dark phase (Fig. 2B). Moreover, mouse sleep is more fragmented than human sleep and does not occur in a consolidated bout as it does in humans. These differences probably result from varied sleep regulatory mechanisms between human and mice which may contribute to differed phenotypes caused by the same genetic mutation. This could at least partly explain the difference in sleep phenotype observed in NPSR1 human mutation carriers (reduced by 2~4 hours during the rest phase) and *Npsr1-Y206H* mice (reduced sleep is mostly confined to the active phase for 1 hour).

Interestingly, homology of *NPSR1* across evolutionary time is less than many genes regulating critical biological processes (e.g. cell cycle regulation or ion channel proteins and cell excitability). The protein sequence identity of hNPSR1to the following species is: mouse (80%), dog (61%), *Xenopus* (51%), zebrafish (20%). This fits with the differences mentioned above in sleep, even from human to mice. Thus, while there is much to learn from studies of sleep and sleep-like behavior in

many model systems, it seems likely that their will interesting differences in human sleep regulation. And it will be interesting to better understand not only the similarities, but also the differences in human sleep vs. other organisms.

In sum, we identified the *NPSR1-Y206H* mutation from the FNSS subjects. Mice carrying the mutation showed similar phenotype to human FNSS. These data support a causal role of the *NPSR1-Y206H* mutation for the human short sleep phenotype. Thus, the NPS/NPSR1 pathway provides a potential therapeutic target to improve human sleep and treat sleep-related disorders.

Materials and Methods

Study Design

The objective of this study was to identify the genetic mutation that underlies the short sleep phenotype of FNSS individuals of kindred #50226. Exome sequencing was performed on DNA from 2 FNSS subjects. Contribution of the *NPSR1-Y206H* mutation to the short sleep phenotype was tested by generating *Npsr1*-Y206H knock-in mice using CRISPR/Cas9. Sleep phenotypes of mutant mice were examined with EEG and video recording. Molecular characterizations, including single cell calcium imaging were carried out to investigate the functional alterations of mutant NPSR1. To measure the effect of short sleep on the memory function of *Npsr1* mutant mice, WT and mutant mice were subjected to contextual fear condition tests with or without sleep deprivation. Similar numbers of *Npsr1*-

Y206H mutant and WT littermates were assigned to each group. The sample size in animal studies was determined based on previous experience with similar animal studies. For each experiment, sample size indicated in the figure legend reflects the number of independent biological replicates. The experimenters were blind to genotype of the animals during behavioral tests, calcium imaging, NPS/SHA injection, protein sample preparation and EEG scoring. We only excluded mice with unreadable EEG signals from data analysis.

Nomenclature

For humans—gene (*NPSR1*), protein (NPSR1). For mouse—gene (*Npsr1*), protein (NPSR1). +/+ refers to wild type animals or unaffected human subjects and +/m refers to heterozygous mutant animals or affected human subjects.

Short sleeper characterization and Identification of candidate gene

Human research subjects for this study are voluntary participants. All human participants signed a consent form approved by the Institutional Review Boards at the University of Utah and the University of California, San Francisco (IRB# 10-03952). Self-reported habitual sleep-wake schedules were obtained during structured interviews by one of the authors (C.R.J., L.J.P). Blood sample collection and DNA preparation were performed as previously described*(16)*.

Exome sequencing

Exome sequencing was performed on DNA from 2 individuals. Omicia Opal 0.10.0 software was used to annotate the potentially causative genes/variants in the exomes of each of the affected individuals (after filtering out common variants, dbSNP MAF > 1%) using the HGMD and OMIM databases. Variant prioritization (using Ingenuity Variant Analysis) included generating a list of all variants, filtered for (1) Co-segregation with the phenotype; (2) Conservation during evolution; (3) Expression of the gene in the CNS; (4) Function of the gene (sleep-relevant, at least neuronal-relevant); (5) Incidence in the population < 0.0001.

Generation of Npsr1-Y206H knock-in and knock-out mice

Npsr1-Y206H knock-in mice were generated by CRISPR/Cas9. Briefly, DNA template for sgRNA was amplified with primers containing T7 promoter and the sgRNA-targeting sequences. Primer sequences are as follows: forward, ‘TTAATACGACTCACTATAGGAGCAATGAATA AGTGTGCAAGTTTTAGAGCTAGAAATAGC’; reverse, ‘AAAAGCACCGACTCGGTGCC’. The sgRNA was then transcribed (MEGAshortscript T7 kit, Life Technologies) and purified (MEGAclear kit, Life Technologies) in RNase-free water. The oligo DNA sequence for recombination is as follows: ‘gtccagcccgtggcctctcacctgataattgccaagggaatgaagtacaccagaaaggcgac gatggtcatgtacggggtccagtgcgagtcatccggccacagtgcccagcactgcacctcaccattggaaagtgt

ccttttcccaaatatgatcagcgtgggaatggaga'.

Super-ovulated female C57BL/6J mice were mated to C57BL/6J stud males, and fertilized zygotes were collected from oviducts. Cas9 protein (50 ng/mL), sgRNA (20 ng/mL) and targeting oligo DNA (20 ng/mL) were mixed and injected into the pronucleus of fertilized zygotes. Injected zygotes were implanted into oviducts of pseudopregnant CD1 female mice. Founders were genotyped by PCR and sequencing. Mice were then crossed with C57Bl/6J mice for at least four generations to dilute out potential off-target affects. Two independent lines were chosen for experiments and gave similar results in all tests, demonstrating that the findings were not due to insertion affects. *Npsr1* knock-out mice were obtained as a by-product when generating the knock-in mice. Two founders were found missing the whole exon encoding Y206 which is predicted to cause frame-shift from Glu160 (371 AAs total).

Animal Studies

All experimental animals were singly housed on a LD 12:12 cycle and given *ad libitum* access to food and water. Male mice were used for all behavioral experiments including ANY-maze, EEG and fear conditioning tests. Mice were at least eight weeks old at the time of surgery. Littermates were used for studies comparing WT and mutant mice. We noticed that mouse sleep behaviors were significantly affected by light intensity. For the experiments that compared the

sleep time and locomotor activity between WT and mutant mice, the light intensity of the room was strictly controlled between 80-100 lux.

All experimental protocols were approved by the University of California, San Francisco IACUC following NIH guidelines for the Care and Use of Laboratory Animals.

ANY-maze monitoring

Mice were kept in individual cages with free access to food and water. Mice were monitored by infrared camera and tracked by an automatic video tracking system (Storlting, Wood Dale, IL; RRID SCR_014289). Mice were entrained to LD 12:12 for 1 week and then locomotor activity was recorded for 3-4 days. Walking distance and mobile times were calculated using ANY-maze software and data were averaged.

EEG/EMG Implantation

Four guide holes were made using a 23-gauge surgical needle placed epidurally over the frontal cortical area (1 mm anterior to bregma, 1 mm lateral to the midline) and over the parietal area (3 mm posterior to bregma, 2.5 mm lateral to midline). One ground screw and three screws with leads were placed into the skull through the holes. The screws with leads were then soldered onto a 6-pin connector EEG/EMG headset (Pinnacle Technologies, Lawrence, Kansas). For EMG

recordings, EMG leads from the headset were placed into the neck muscle. The headset was then covered with black dental cement to form a solid cap atop the mouse's head. The incision was then closed with VetBond (3M, Santa Cruz Biotech) and animals were given a subcutaneous injection of marcaine (0.05 mg/kg) prior to recovery on a heating pad. Behavioral experiments were conducted 3 weeks later to allow for sufficient recovery and for viral expression.

EEG/EMG Recording and Scoring

For EEG/EMG recording, mice were singly-housed and habituated to the recording cable for 7 days in LD 12:12. Tethered pre-amplifiers were attached to the headset of the mice. The signals were relayed through commutators that allowed the animal to move freely. Data was acquired through the Sirenia software package (Pinnacle Technologies, Lawrence, KS) *(48)*.

Sleep was scored semi-automatically with Sirenia Sleep Pro software in 10-s epochs for wakefulness, NREM, and REM sleep, and then subsequently hand-scored by researchers blinded to genotype with the assistance of spectral analysis using Fast Fourier Transformation. In general, wakefulness was defined as desynchronized low-amplitude EEG and heightened tonic EMG activity with phasic bursts. NREM sleep was defined as synchronized, high-amplitude, low-frequency (0.5–4 Hz) EEG and substantially reduced EMG activity compared with wakefulness. REM sleep was defined as having a pronounced theta rhythm (4–9 Hz) with no EMG

activity.

To examine sleep-wake behavior under baseline conditions, EEG/EMG signals were recorded and analyzed for the entire two consecutive days from the onset of the light phase. Sleep (NREM and REM sleep) time and power spectrum were averaged data from two consecutive days. For sleep deprivation, mice were sleep deprived for 6 hours from the onset of the light phase by gently touching when they started to recline and lower their heads. Food and water were available. EEG/ EMG signals were recorded and analyzed for the entire 18 hours following sleep deprivation.

For spectral analysis, artifacts and state transition epochs were excluded. Relative NREM EEG power spectra were calculated at a 0.1 Hz resolution. Individual differences were normalized by expressing each frequency bin as a percentage of total EEG power over a 24-hour period for each mouse. As various behavioral states tend to have different EEG power- which could affect total power depending on each individual's relative time spent in each state- the relative contribution to total power of each state was weighted by the respective time spent in that state *(49)*. The time course of delta power (1.0-4.0 Hz) in NREM sleep was computed as previously described *(22, 49–51)*. Change of NREM sleep delta power across light-dark cycle is determined by the delta band of NREM sleep and normalized to the average NREM sleep delta power during ZT9-12 of the baseline recording day *(52, 53)*. In the dark phase, especially early dark phase, NREM sleep is absent in some time points. As a result, delta power is not presented for every hour. Intervals were chosen so that every mouse had NREM sleep for the time point shown in the

figure.

Stereotaxic Viral Injection

Animals were anesthetized with 2% isoflurane and placed in a stereotaxic head frame on a heating pad. Opthalmic ointment was applied to the eyes to prevent drying. A midline incision was made down the scalp and a craniotomy was made using a dental drill. A 10 µl Nanofil Hamilton syringe (WPI, Sarasota, FL) with a pulled glass needle was used to infuse virus with a microsyringe pump (UMP3; WPI, Sarasota, FL) and its controller (Micro4; WPI, Sarasota, FL). Virus was infused at a rate of 50 nl/min. Following infusion, the needle was kept at the injection site for 10 min and then slowly withdrawn at 0.01 mm/sec. All stereotaxic coordinates are relative to bregma. AAV1/Syn-GCaMP6f.-WPRE-SV40 was injected into the CMT (−1.5 mm anteroposterior (AP); 0.0 mm mediolateral (ML); −3.7 mm dorsoventral (DV)) or LH (−1.5 mm anteroposterior (AP); 1.0 mm mediolateral (ML); −5.0 mm dorsoventral (DV)) with a total of 300 nl of virus.

Intracerebroventricular (i.c.v.) Injection

Prior to drug injections, mice were anesthetized with 2% isoflurane and placed in a stereotaxic head frame on a heating pad. For each mouse, the bregma was located without exposure of the skull*(54)*. A guarded 23-gauge needle was used to punch a

hole 0.2 mm posterior to the bregma and 1.0 mm lateral to the midline. The Nanofil syringe (WPI) was used to inject NPS (#5857, Tocris) (0.1 nmole or 1 nmole in 2 uL saline) or vehicle (saline) into the right cerebral ventricle at a depth of 2.5 mm from the skull at ZT11. Mice were allowed to recover for 5 min and then placed back in the home cage. Vehicle or SHA 68 (SML1459-25MG, Sigma) (50 mg/kg in PBS, 10% cremophor EL) were injected (i.p.) 10 min before NPS. Brain tissues were collected 1 hour after the i.c.v. injections.

Calcium imaging in explants

Male *Npsr1* mice (either WT or mutant, 8~12 weeks) were infused with AAV1/Syn-GCaMP6f.-WPRE-SV40 virus (300nl) into the CMT or LH using the coordinates described above 2-4 weeks before slice preparation. Slices were prepared following the previously reported protocol*(55)*. Briefly, animals were anesthetized under isoflurane and briefly perfused intracardially with 10 ml of ice-cold NMDG solution (92mM NMDG, 30mM $NaHCO_3$, 25 mM glucose, 20mM HEPES, 10mM $MgSO_4$, 5mM sodium ascorbate, 3mM sodium pyruvate, 2.5mM KCl, 2mM thiourea, 1.25mM NaH_2PO_4, 0.5mM $CaCl_2$ (pH 7.3, 300 mOsm, bubbled with 95% O_2 and 5% CO_2)). The brains were then quickly removed and placed into additional ice-cold NMDG solution for slicing. Coronal slices were cut using a Leica VT1200S vibratome at 300 µm thickness, and warmed to 36.5°C for 10 min. Slices were transferred to

room temperature (22–24°C) HEPES holding solution containing 92mM NaCl, 30mM $NaHCO_3$, 25mM glucose, 20mM HEPES, 5mM sodium ascorbate, 3mM sodium pyruvate, 2.5mM KCl, 2mM thiourea, 2mM $MgSO_4$, 2mM $CaCl_2$, and 1.25mM NaH_2PO_4, (pH 7.3, 300 mOsm, bubbled with 95% O_2 and 5% CO_2) for 1 - 2 hr.

After incubation, slices were transferred to the recording chamber and constantly perfused with room-temperature (22–25°C) recording solution containing 119mM NaCl, 2.5mM KCl, 1.25mM NaH_2PO_4 , 24mM $NaHCO_3$, 12.5mM glucose, 2mM $CaCl_2$, and 2mM $MgSO_4$ (pH 7.3, 300 mOsm, bubbled with 95% O_2 and 5% CO_2) at a rate of 4 ml per minute.

An integrated microscope (nVista HD, Inscopix) was used to image the GCaMP signal from the slice. We chose this small microscope because the lens that collects the light can go directly into the perfusion buffer from above to image the surface-layer cells, which are usually healthier and more accessible to the drug. In addition, the recording and data processing software are commercially available and user-friendly. Although the resolution of the images might be lower than those gathered by some confocal microscopes, it is sufficient for our purposes here. We used the data acquisition software (nVista, Inscopix) to acquire the images (a range of 6%–10% of LED intensity, Gain 2-3, 2.5-5fps). During each recording, the recording solution containing NPS (1μM) was turned on and off at the indicated time point.

The video was then analyzed by the Inscopix Data Processing (Inscopix)

software. Each video was processed with spatial crop, spatial filter, motion correction and dF/F calculation. Regions of interest (ROIs, considered as a single cell) were manually selected based on the shape and dF/F changes throughout the recording. ROIs that exhibited short bursts of dF/F changes or fluctuations during the recording were analyzed. ROIs that showed steady decreases, increases or maintained constant ΔF/F were excluded from further analysis. The dF/F changes were then aligned with the time window of NPS treatment and the cells were categorized into four groups based on the alignment. Researchers were blinded to the genotype of the slice when processing and analyzing the data to ensure the same criteria applied to calculate both the WT and mutant cells

Protein Extraction and Immunoblot Analysis

Cortex and deep brain structures (striatum, thalamus, hypothalamus) of wild type and mutant brains were dissected in ice-cold PBS treated with protease and phosphatase inhibitors (#11697498001 and #5892970001, Roche). Protein was extracted by homogenizing the tissues with 2mL RIPA buffer (10 mM Tris-HCl (pH 7.4 - 7.6), 150 mM NaCl, 1mM EDTA, 0.1% sodium deoxycholate, 1mM EDTA, 1% NP-40, proteinase inhibitor, phosphatase inhibitor). Western blotting was performed according to standard procedures using the corresponding antibodies. Antibodies were used at concentrations recommended by the manufacturer. Antibodies used in this study included anti-CREB (phospho S133) (#9198, Cell

signaling), anti-CREB (#9197, Cell signaling), anti-EF2 (phospho T56/T58) (ab82981, Abcam), anti-EF2 (#2332, Cell Signaling), anti-synapsin-1 (phospho S605) (#88246, Cell Signaling), anti-synapsin-1 (sc8295, Santa Cruz). Band intensities were determined using Image J software (NIH).

RNA Isolation and Real-Time PCR

Total RNA was isolated from frozen tissues (brain) with Trizol reagent (Thermo Fisher Scientific). A total of 5ug total RNA was reverse transcribed using the Superscript IV Kit (Thermo Fisher Scientific). cDNA was then quantified using SYBR green real-time PCR analysis with the QuantStudio 6 Flex Real-Time PCR System (Thermo Fisher Scientific). The real-time PCR data were normalized to *Actb*.

Contextual fear conditioning

Mice were handled gently for 2 min/day for 5 consecutive days prior to each experiment and were placed in the experiment room one hour prior to training or test to acclimate to the new environment. On the day of training, at either ZT3 or ZT23, mice were allowed to explore the conditioning chamber for 3 min prior to two (ZT3) or three (ZT23) 2sec 0.6mA foot-shocks with 1min interval. Animals were left in the chamber for an additional 1min following the shock and then returned to their home cage. 24 hours post-training, in order to test mice at the same time of day, mice were returned to the training chamber for 5min and freezing responses

were measured. Freezing responses were analyzed using automated tracking software and expressed as a percentage of total time spent in the testing chamber. In the sleep deprivation group, mice were subjected to 6-hour sleep deprivation following training.

Statistical analysis

The following methods were used to determine statistical significance: unpaired *t* test, one-way ANOVA, two-way ANOVA and Chi-square test. Unless otherwise stated, all values are presented as means ± SEM. Original data are provided in data file S1.Data is judged to be statistically significant when $P < 0.05$. In figures, asterisks denote statistical significance * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$. All statistical analysis was performed using GraphPad PRISM 7 software.

Supplementary Materials

Fig. S1. Generation of *Npsr1-Y206H* mice.

Fig. S2. Sleep/wake measurements in *Npsr1-Y206H* and *Npsr1* knockout mice.

Fig. S3. EEG data analysis of sleep/wake behavior of *Npsr1-Y206H* mice.

Fig. S4. High sleep pressure was observed in *Npsr1-Y206H* mice.

Fig. S5. Hyper-phosphorylated SNIPPs were observed in *Npsr1-Y206H* mice following SD.

Fig. S6. Wake and sleep questionnaire for FNSS.

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Figure Legends

Fig. 1. *NPSR1-Y206H* mutation was identified in a natural short sleep family. (**A**) Pedigree of the family (K50226) carrying the *NPSR1* mutation. The self-reported total sleep time per 24-hour day of mutation carriers is indicated. (**B**) NPSR1-Y206H is localized to the extracellular domain between transmembrane domains 4 and 5 (TM4/5) and is highly conserved among vertebrate NPSR1 orthologs. (**C**) Schematic of the mutations (Y206H and N107I) in the extracellular loops of NPSR1.

Fig. 2. *Npsr1-Y206H* mice demonstrate reduced sleep time. (**A**) Mouse

movement was tracked by ANY-maze under LD 12:12. Total mobile time in 24 hours, light phase, and dark phase were calculated in *Npsr1*+/+ (N = 19) and *Npsr1*+/m (N = 15) mice. (**B** to **D**) Total sleep (B), NREM sleep (C) and REM sleep (D) time within 24 hours, light phase and dark phase measured by EEG/EMG were calculated in *Npsr1*+/+ (N = 14) and m/+ (N = 12) mice. (**E**) NREM and REM sleep time were plotted hourly over 24 hours in *Npsr1*+/+ (N = 14) and m/+ (N = 12) mice. (**F**) NREM sleep delta power normalized to the average value during ZT9-12 was plotted hourly in *Npsr1*+/+ (N = 14) and +/m (N = 12) mice over 24 hours. **P* < 0.05, ***P* < 0.01, ****P* < 0.001, *****P* < 0.0001, two-tailed Student's *t*-test (A to D); Two-way RM ANOVA, post-hoc Sidak's multiple comparisons test (E and F). Data are mean ± SEM.

Fig. 3. EEG data analysis for sleep/wake measurements in WT and *Npsr1-Y206H* mice after sleep deprivation. (**A**) NREM sleep delta power after SD (ZT0-6) normalized to the average NREM delta power during ZT9-12 of the baseline recording were plotted every hour in *Npsr1*+/+ (N = 14) and +/m (N = 12) mice for 18 hours. (**B** to **D**) Total (B), NREM (C) and REM (D) sleep were calculated during indicated time periods for *Npsr1*+/+ (N = 14) and +/m (N = 12) mice after 6 hours of SD (ZT0-6). (**E**) Cumulative NREM and REM sleep loss and gain compared with baseline conditions for the sleep deprivation experiment in *Npsr1*+/+ (N = 14) and +/m (N = 12) mice. **P* < 0.05, ***P* < 0.01, ****P* < 0.001, two-tailed Student's *t*-test

(B to D left panel); Two-way RM ANOVA, post-hoc Sidak's multiple comparisons test (B to D right panel, A and E). Data are mean ± SEM.

Fig. 4. Mutant NPSR1 is more active *in vivo*. (**A**) p-CREB immunoblots for brain lysates collected at indicated time points from *Npsr1*+/+ and +/m mice. (**B**) Quantified results of (A) from *Npsr1*+/+ (ZT2, N = 4; ZT11, N = 5; ZT22, N = 4) and +/m mice (ZT2, N = 4; ZT11, N = 5; ZT22, N = 3). (**C**) Immunoblots for brain lysates collected at ZT11 from *Npsr1* +/+ and +/m mice after different doses of NPS. (**D**) Quantified results of (C) from *Npsr1*+/+ (Saline, N = 4; 0.1nmol NPS, N = 3; 1nmol NPS, N = 4) and +/m mice (Saline, N = 4; 0.1nmol NPS, N = 3; 1nmol NPS, N = 3). (**E**) Western blot of p-CREB expression in WT brain lysates collected at ZT11 after SHA 68 (i.p.) or/and NPS (i.c.v.) injection. Group sizes: Veh / SHA 68 (50 mg/kg) + Saline, N = 3; Veh / SHA 68 (50 mg/kg) + NPS (1 nmol), n = 4. (**F**) Immunoblots for brain lysates collected at ZT11 from *Npsr1*-/- mice after saline (N = 4) or 1nmol NPS injection (N = 4). Quantified results are shown in panels on the right. *$P < 0.05$, ***$P < 0.001$, ****$P < 0.0001$, ns = not significant. Two-way ANOVA, Sidak's multiple comparisons test (B and D). One-way ANOVA, Dunnett's multiple comparisons test (E). Two-tailed Student’s *t*-test (F). Data are mean ± SEM.

Fig. 5. Calcium imaging of CMT neurons shows increased activity for one

sub-type in *Npsr1-Y206H* mice. (**A**) Schematic of calcium imaging set up for recording the activity of CMT neurons in brain slices. (**B**) Schematic of anatomy for CMT. The injection/recorded area is marked with a pink circle. (**C**) Representative GCaMP fluorescence traces in different categories of cells that responded differentially to NPS treatment. Group1: pulse activation; Group2: fast and long-lasting activation; Group 3: fast activation and recovery; Group 4: inhibition. (**D**) Percentage of cells that show different types of response to NPS treatment in *Npsr1*+/+ (N = 8), *Npsr1*+/m (N = 7) and *Npsr1*-/- (N = 3) brain slices. **P* < 0.05, **** *P* < 0.0001, ns = not significant; Chi-square test (D). n = number of cells; N= number of animals.

Fig. 6. Contextual memory of *Npsr1-Y206H* mice is resistant to sleep loss. (**A** and **B**) Mice were trained in contextual fear conditioning at the beginning of the light phase (A) or at the end of the dark phase (B). Freezing response in the trained context was tested 24 (day 1) and 48 (day 2) hours after training. Percentage of time freezing during the 5 min of the test without SD (A, *Npsr1*+/+ (N = 17) and +/m (N = 12); B, *Npsr1*+/+ (N = 12) and +/m (N = 11)) or with SD (A, *Npsr1*+/+ (N = 10) and +/m (N = 10); B, *Npsr1*+/+ (N = 10) and +/m (N = 11)) is shown in box-and-whisker plots. **P* < 0.05, *** *P* < 0.001, ns = not significant. One-way ANOVA multiple comparisons followed by Tukey’s multiple comparisons test.