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Involvement of Ca²⁺-Dependent Hyperpolarization in **Sleep Duration in Mammals**

Highlights

- A simple model predicts Ca²⁺-dependent hyperpolarization regulates sleep duration
- Impaired/enhanced Ca²⁺-dependent hyperpolarization decreases/increases sleep duration
- Impaired Ca²⁺-dependent hyperpolarization increases neural excitability
- Impaired Ca²⁺/calmodulin-dependent kinases (Camk2a/ Camk2b) decreases sleep duration

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In Brief

Tatsuki et al. present that a Ca2+dependent hyperpolarization pathway underlies the regulation of sleep duration in mammals. They predicted the hypothesis by a simple averaged-neuron model and verified it by phenotyping 21 KO mice and whole-brain imaging with pharmacological intervention.





Involvement of Ca²⁺-Dependent Hyperpolarization in Sleep Duration in Mammals

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SUMMARY

The detailed molecular mechanisms underlying the regulation of sleep duration in mammals are still elusive. To address this challenge, we constructed a simple computational model, which recapitulates the electrophysiological characteristics of the slowwave sleep and awake states. Comprehensive bifurcation analysis predicted that a Ca²⁺-dependent hyperpolarization pathway may play a role in slowwave sleep and hence in the regulation of sleep duration. To experimentally validate the prediction, we generate and analyze 21 KO mice. Here we found that impaired Ca2+-dependent K+ channels (Kcnn2 and Kcnn3), voltage-gated Ca2+ channels (Cacna1g and Cacna1h), or Ca2+/calmodulin-dependent kinases (Camk2a and Camk2b) decrease sleep duration, while impaired plasma membrane Ca²⁺ ATPase (Atp2b3) increases sleep duration. Pharmacological intervention and whole-brain imaging validated that impaired NMDA receptors reduce sleep duration and directly increase the excitability of cells. Based on these results, we propose a hypothesis that a Ca²⁺-dependent hyperpolarization pathway underlies the regulation of sleep duration in mammals.

INTRODUCTION

The invention of the electroencephalogram (EEG) more than 85 years ago (Berger, 1929; Caton, 1875) enabled the global characterization of the mammalian brain's electrical behavior. During sleep, an EEG mostly displays high-amplitude low-frequency fluctuations (Walter, 1937), which are generated by synchronized slow oscillations of the cortical neuron membrane potential; during waking, an EEG exhibits low-amplitude high-frequency fluctuations, which are generated by the irregular firings of cortical neurons (Steriade and McCarley, 2005). These two states are mutually exclusive in a normal brain, and their ratio is homeostatically regulated (Steriade and McCarley, 2005). For example, sleep pressure (or the need for sleep) is high after sleep deprivation and gradually decreases during sleep. Importantly, sleep pressure can be measured as an EEG slow-wave activity, i.e., an EEG delta power (Borbély et al., 1981; Nakazawa et al., 1978; Webb and Agnew, 1971). However, the detailed molecular mechanisms underlying mammalian sleep/wake cycles are still elusive.

To elucidate the complex dynamics of sleep/wake cycles, a number of computational models have been proposed. The two-process model attempts to capture the relatively slow and macroscopic dynamics of sleep by describing two regulatory processes, S and C, which are respectively a sleep-dependent homeostatic process and a sleep-independent circadian process (Borbély, 1982). Process S represents the sleep pressure, which increases in proportion to the quantity and quality of the preceding awake state and decreases in the subsequent sleep state; process C is sleep regulation by the circadian clock, which has been molecularly identified in fly and mammals by forward genetics (Borbély, 2001; Lowrey and Takahashi, 2011). Although the two-process and similar models can explain the slow and macroscopic dynamics of sleep/wake cycles, they are too simple to predict the details underlying molecular mechanisms, particularly of the homeostatic process S, which remain elusive.

Another series of computational models were derived to explain the relatively fast and microscopic dynamics of experimentally observed electrophysiological behavior. The highamplitude low-frequency oscillations are observed by EEG and by intracellular and extracellular electrophysiological recording during slow-wave sleep (SWS), which has two electro-physiologically different phases: the depolarized, bursting phase and the hyperpolarized, silent phase (Steriade, 2003; Steriade et al., 2001). Several computational models for these phases, based on populations of interacting neurons, have been proposed (Bazhenov et al., 2002; Chen et al., 2012; Compte et al., 2003; Hill and Tononi, 2005; Sanchez-Vives et al., 2010;



Figure 1. The Averaged-Neuron Model Predicts that a Ca²⁺-Dependent Hyperpolarization Pathway Plays a Pivotal Role in Slow-Wave Sleep (A) Schematic diagram of the AN model. Light red: extrinsic ion channels. Light blue: intrinsic ion channels and Ca²⁺-pumps/exchangers. (B) Representative SWS firing patterns and intracellular Ca²⁺ concentration.

(legend continued on next page)

Timofeev et al., 2000). These "neuron-network" models elegantly recapitulate the behavior of cortical and thalamic neurons. These studies indicate that the transition from bursting to silent phase is facilitated by (1) the enhancement of Ca^{2+} and Na^+ -dependent K⁺ channels in pyramidal neurons, (2) the inhibition of the excitatory synaptic current between pyramidal neurons, or (3) the enhancement of feedback repression among pyramidal neurons and interneurons.

To date, however, genetic evidence for these mechanisms has been lacking especially in mammals. Moreover, the simulated slow-wave oscillation models are heavily dependent on the given parameter values, and thus their predictive power is limited. In part, this is because the neuron-network models possess such a huge parameter space that the number and type of parameter sets (and a common critical pathway) that can generate slow-wave oscillations cannot be identified comprehensively. A simple, but not simplistic, computational model is required to elucidate the underlying mechanisms of sleep/wake cycles.

Various hypotheses have been proposed to connect fast/microscopic and slow/macroscopic dynamics, but they have not been fully integrated into computational models. The "sleepinducing substance (SIS)" hypothesis (or "sleep-regulatory substance" hypothesis, in general) (Clinton et al., 2011; Krueger et al., 2008; Obal and Krueger, 2003), in which SISs accumulate during waking and gradually degrade during sleep, has existed since 1909 (Ishimori, 1909). In the SIS hypothesis, process S is intuitively represented as the concentration of SISs (Factor S). Several candidate molecules for the SISs have been identified including adenosine, nitric oxide, prostaglandin D, tumor-necrosis factor, interleukin 1, and growth-hormone-releasing hormone. All of these molecules can induce sleep when injected into animals. However, it remains difficult to explain fast electrophysiological dynamics using the slow timescale observed for the accumulating SISs. Therefore, it is important to identify the molecular targets of SISs to understand the shift between slow and fast dynamics.

Alternatively but not exclusively, the "synaptic homeostasis" hypothesis proposes that synaptic strength of cortical neurons represent process S (Tononi and Cirelli, 2006). For example, during waking, synaptic strength increases via learning processes such as long-term potentiation, whereas during much of sleep, synaptic strength decreases. Hence, the synaptic homeostasis hypothesis attempts to connect the fast dynamics of the cortical neurons' membrane potential (slow-wave oscillations) with the relatively slow dynamics for process S (synaptic strength). Although some experimental results support the synaptic homeostasis hypothesis (Vyazovskiy et al., 2008), others challenge it (Yang et al., 2014). In addition, computational neuron-network models apparently contradict this hypothesis, because

the enhancement of excitatory synaptic current between pyramidal neurons (i.e., increased synaptic strength) in the current neuron-network models enhances the awake rather than the sleep state, by depolarizing the pyramidal neurons. Hence, we need a computational model that is compatible with the relatively slow homeostatic dynamics (e.g., of process S) and the relatively fast electrophysiological dynamics (e.g., slow-wave oscillations).

RESULTS

The Computational Averaged-Neuron Model Recapitulates Slow-Wave Sleep Firing Patterns

To reproduce the oscillating bursting and silent phases of cortical neurons during slow-wave sleep, we simplified the neuron-network models (Bazhenov et al., 2002; Chen et al., 2012; Compte et al., 2003; Hill and Tononi, 2005; Sanchez-Vives et al., 2010; Timofeev et al., 2000). We first performed mean-field approximations of a population of neurons to construct an "averaged-neuron" model (AN model) (Figure 1A; Table S1). We assumed, as a first-order approximation, that the averaged neuron could interact with itself (directly or indirectly) through excitatory (Na⁺ currents mediated by AMPA receptors, IAMPA and Ca²⁺ currents mediated by NMDA receptors, I_{NMDA}), or inhibitory (CI⁻ currents mediated by GABA_A receptors, I_{GABA}) synaptic currents. In addition to extrinsic currents, we incorporated, as intrinsic currents, depolarizing Na⁺ and Ca²⁺ currents and hyperpolarizing K⁺ currents. The depolarizing Na⁺ and Ca^{2+} currents are mediated by voltage-gated (I_{Na}) or the persistent (I_{NaP}) Na⁺ channels and voltage-gated Ca²⁺ channels (I_{Ca}), respectively, whereas the K⁺ currents are mediated by several types of K⁺ channels, including voltage-gated ($I_{\rm K}$), leak ($I_{\rm L}$), fast A-type (I_A), inwardly rectifying (I_{AR}), slowly inactivating (I_{KS}), and Ca^{2+} dependent (I_{KCa}). We also assumed that Ca^{2+} pumps/ exchangers turn over Ca²⁺ with the time constant, τ_{Ca} . Notably, the AN model recapitulated the SWS firing patterns, with its alternating bursting and silent phases (Figure 1B). Moreover, the parameter values in the AN model that generated slow-wave oscillations were similar to those in neuron-network models, implying that sufficient information to generate SWS was preserved in this simple model.

Close inspection showed that the SWS firing patterns in the AN model were characterized by a bursting phase, in which Ca^{2+} enters the cell mainly through the NMDA receptor (NMDAR) and voltage-gated Ca^{2+} channels (Ca_v channels), followed by a silent phase, in which accumulated Ca^{2+} evokes Ca^{2+} -dependent K⁺ channels (K_{Ca} channels) (Figure 1B; Figure S1A). It then exits the cell through Ca^{2+} pumps/exchangers with τ_{Ca} (Figure 1A). To investigate the role of this Ca^{2+} -dependent hyperpolarization pathway in the SWS firing patterns, we performed a

⁽C) The downregulation of the conductance of K_{Ca} channels (g_{KCa}), Ca_v channels (g_{Ca}), and NMDARs (g_{NMDA}) and the time constant for Ca²⁺ efflux (τ_{Ca}) result in the transitions from SWS (red) to awake (blue) firing patterns.

⁽D) The workflow of a random parameter search to identify parameter sets that result in SWS firing patterns.

⁽E) The normalized membrane potential and intracellular Ca²⁺ are shown for all 1,113 parameter sets yielded by the parameter search.

⁽F) Bifurcation analysis of single parameter sets in 1,113 parameter sets that elicited SWS firing patterns.

⁽G–I) Summary of bifurcation analysis under WT (G), NMDAR KO (H), or Ca_v channel KO (I) conditions. Red text indicates the conductance and time constant related to the Ca²⁺-dependent hyperpolarization pathway.

bifurcation analysis (i.e., a gradual change in parameter values) of the conductance of each intrinsic and extrinsic current and of the Ca²⁺ efflux rate. As expected, we found that downregulating the conductance of the NMDAR (g_{NMDA}), Ca_v channels (g_{Ca}), K_{Ca} channels (g_{KCa}), and/or reducing the time constant (τ_{Ca}) of Ca²⁺ efflux mediated by Ca²⁺ pumps/exchangers caused the transition from SWS to awake firing patterns (Figure 1C; Figures S1B–S1G), implying that this Ca²⁺-dependent hyperpolarization pathway may have a pivotal role in regulating the SWS firing patterns.

The Averaged-Neuron Model Predicts that a Ca²⁺-Dependent Hyperpolarization Pathway May Play a Role in Slow-Wave Sleep and hence in the Regulation of Sleep Duration

To investigate the generality of these findings, we sought to identify a broad variety of parameter sets that could generate SWS firing patterns (Figure 1D). We comprehensively searched for the conductance of the intrinsic (0.01–100 mS/cm²) and extrinsic (0.002–20 μ S) currents and the time constant for Ca²⁺ efflux (10–1,000 ms) that resulted in this pattern. In this search, we generated more than 10,000,000 random parameter sets from exponential distributions. From these, we identified 1,113 parameter sets that generated SWS firing patterns. The success rate was $\sim 5.7 \times 10^{-3}$ %. These patterns exhibited intracellular Ca²⁺ waves that were coupled with the membrane potential (Figure 1E).

If a particular molecular mechanism is targeted by the homeostatic process S, it should control, directly or indirectly, the transitions between the SWS and awake state, because the accumulation of factor S (or the increase of its guality) presumably facilitates the transition from the awake state to SWS, while its degradation (or the decrease of its guality) enhances the transition from SWS to the awake state. To identify potential targets for factor S, we performed a comprehensive bifurcation analysis of the 1,113 SWS parameter sets and searched for channel conductance or the time constant that could contribute to the transition from SWS to awake firing patterns. In this analysis, each channel conductance or the time constant was gradually changed within the range from 10^{-3} to 10 times its value in the original parameter set (Figure 1F). Indeed, downregulation of the Ca²⁺-dependent hyperpolarization pathway led to the transition from slow-wave-sleep to awake firing patterns in 848 (76.2%), 581 (52.2%), and 861 (77.4%) cases with altered g_{KCa} , g_{NMDA} , and τ_{Ca} , respectively (Figure 1G). These results were consistent with the suggested relationship between cortical delta waves and K_{Ca} conductance (Steriade et al., 1990) and also predicted that an impairment of K_{Ca} channels or NMDARs, or an enhancement of Ca²⁺ pumps/exchangers would facilitate the transition from SWS to awake firing patterns and hence decrease the sleep duration (i.e., impairment of Ca²⁺pump/exchanger would increase the sleep duration).

To verify the role of the Ca²⁺-dependent hyperpolarization pathway in SWS, we conducted the parameter searches under the condition of knocked out (KO) K_{Ca} channels ($g_{KCa} = 0$) or NMDARs ($g_{NMDA} = 0$). The success rate of the K_{Ca} channel KO parameter search was severely decreased (~1.1 × 10⁻⁵%) compared to the wild-type (WT) parameter search, suggesting that K_{Ca} channels are important for generating SWS firing patterns, whereas the success rate of the NMDAR KO parameter search was not dramatically reduced (Figure S1L). Since the intracellular Ca²⁺ oscillated together with the membrane potential of SWS firing patterns in the NMDAR KO search (Figure S1H), we speculated that there might be an alternative Ca²⁺ entry mechanism. Indeed, bifurcation analysis revealed that changes in g_{Ca} led to the transition more often (836 cases, 71.1%, Figure 1H) in the NMDAR KO search than in the WT parameter search (108 cases, 9.7%, Figure 1G).

Therefore, we next conducted the parameter search under the double KO of Ca_v channels ($g_{Ca} = 0$) and NMDARs ($g_{NMDA} = 0$) to confirm the redundant role of these Ca2+ entry mechanisms. In fact, the success rate of the parameter search was severely decreased ($\sim 9.2 \times 10^{-5}$ %) compared to the WT parameter search (Figure S1L). We also noted that the success rate was not reduced under the Cay channel single KO search (Figure S1L) and that these SWS firing patterns were also accompanied by intracellular Ca2+ waves (Figure S1I). As expected, we found that the downregulation of NMDARs (g_{NMDA}) under the Ca_v channel KO condition more often (895 cases, 88.5%, Figure 1I) led to the transition than in the WT parameter search (581 cases, 52.2%, Figure 1G). Overall, these results suggested that Ca_v channels and NMDARs have redundant roles in Ca2+ entry to generate SWS firing patterns and hence that the Ca²⁺-dependent hyperpolarization pathway is involved in SWS. We also found that there were no marked changes in the success rate $(\sim 7.6 \times 10^{-3}\%)$ or bifurcation analysis when we imposed the KO of GABAA receptors on the random parameter search (Figures S1J and S1K). This result is consistent with a previous report, in which the hyperpolarization during SWS is not mediated by GABAergic inhibition (Steriade et al., 2001). From these bifurcation analyses, we predicted that impairment of ion channels related to the Ca²⁺-dependent hyperpolarization pathway might decrease the sleep duration by having the cortex tend to generate awake firing patterns than SWS firing patterns. On the other hand, we predicted the impairment of Ca²⁺-pump/ exchanger would increase the sleep duration by facilitating the Ca²⁺-dependent hyperpolarization pathway.

Impairment of Ca²⁺-Dependent K⁺ Channels Decreases Sleep Duration

To validate the AN model, we first tested the prediction that downregulation of K_{Ca} channels would decrease the sleep duration. In the mouse genomes, eight genes (K_{Ca} 1.1 [Kcnma1], K_{Ca} 2.1 [Kcnn1], K_{Ca} 2.2 [Kcnn2], K_{Ca} 2.3 [Kcnn3], K_{Ca} 3.1 [Kcnn4], K_{Ca} 4.1 [Kcnt1], K_{Ca} 4.2 [Kcnt2], and K_{Ca} 5.1 [Kcnu1]) are categorized into the K_{Ca} channel family according to their sequence similarity (Wei et al., 2005).

To knock out all of the K_{Ca} channel family members, we used the "triple-target CRISPR" method (Sunagawa et al., 2016) (Figures S2A–S2H; Table S2). We also used highly accurate sleep/wake recording system called the Snappy Sleep Stager (SSS) (Sunagawa et al., 2016). We noted that there was no significant influence on sleep phenotype of the triple-target CRISPR itself by creating *Tyrosinase* (*Tyr*) KO mice (Sunagawa et al., 2016). Sleep/wake phenotyping was performed for animals with KO genotype, which was confirmed by qPCR and/ or genomic sequencing (Figure S3; Tables S3 and S4). As



Figure 2. Impairment of Ca²⁺-Dependent K⁺ **Channels Decreases Sleep Duration**

(A) Sleep duration (per hour) over 6 days for KO mice of eight K_{Ca} channels.

(B) Sleep duration (per hour) over 24 hr, averaged over 6 days in the KO mice of K_{Ca} channels. Red lines: the mean sleep duration at each time of day for each strain. Gray: WT (n = 108). Shaded area: SEM for each time point.

0

(C) Distributions of sleep/wake parameters of the KO mice of K_{Ca} channels. Histogram: WT (n = 108). Black dashed line and gray shade: the mean and 1 SD range from the recording of WT.

(D-F) Sleep-duration phenotype for another group of Kcnn2 and Kcnn3 KO mice (set 2). Sleep duration (per hour) over 6 days (D), sleep duration (per hour) over 24 hr, averaged over 6 days (E), and distributions of sleep/wake parameters (F) are shown

(G and H) Sleep-duration phenotype based on EEG/EMG for WT (n = 4) and Kcnn2 KO mice (set 1, n = 9). Sleep duration (per hour) over 6 days (G) and sleep duration, NREM sleep (SWS) duration, and REM sleep duration for 1 day are shown in bar graph (H).

(I) Sleep-duration phenotype based on EEG/EMG for sleep-deprived WT (n = 3) and Kcnn2 KO mice (set1, n = 7). Sleep duration, NREM sleep (SWS) duration, and REM sleep duration for ZT12-ZT24 are shown in bar graph.

WT, C57BL/6N male mice. Error bars, SEM, *p < 0.05, **p < 0.01, ***p < 0.001, see also Supplemental Experimental Procedures for details.

predicted, Kcnn2, Kcnn3, Kcnn4, Kcnt1, and Kcnu1 KO mice exhibited significantly decreased sleep duration (Figures 2A and 2C). The Kcnn2 KO mice caused the largest decrease in sleep duration, i.e., the strongest short-sleeper phenotype, 606.3 ± 14.7 min (mean \pm SEM, n = 5), which was 129.3 min (~2.6 SD) shorter than that of WT mice (p < 0.001). Kcnn3 KO mice also exhibited a strong short-sleeper phenotype, 629.1 ± 11.8 min (mean \pm SEM, n = 6), which was 106.6 min (~2.1 SD) shorter than that of WT mice (p < 0.001). In fact, the Kcnn2 and Kcnn3 KO mice exhibited shorter sleep durations than WT mice over most times of the day (Figure 2B). Both of these mutants displayed lower P_{ws} (the transition probability that an awake state will switch to a sleep one) but a normal P_{sw} (the transition probability that a sleep state will switch to an awake one) compared with WT mice (Figure 2C), indicating that their short-sleeper phenotype was caused by an increase in awake-state stabilization (i.e., longer episode durations of an awake state) but not by a decrease in sleep-state stabilization. We note that Kcnn2 KO mice were previously reported to have a (not-significant) tendency of decrease in sleep duration (Cueni et al., 2008). Our non-invasive sleep phenotyping clearly revealed the significant short-sleeper phenotype in Kcnn2 KO mice. As further validation, we generated another group of Kcnn2 and Kcnn3 KO mice by an independent CRISPR/Cas9 probe set (set 2, Figures S2J and S2K; Table S2) and confirmed the observed shortsleeper phenotypes (Figures 2D-2F). These results strongly suggested that the observed short-sleeper phenotype of Kcnn2 and Kcnn3 KO mice cannot be attributed to the possible off-target effect of CRISPR, but rather to the common genomic defects in Kcnn2 and Kcnn3 gene, respectively.

To exclude the possibility that a gene KO might affect the respiration phenotype and hence that SSS system could miscalculate their sleep duration, we conducted the basal EEG/EMG recording of Kcnn2 KO mice. As a result, the observed shortsleep phenotype of Kcnn2 KO mice was also confirmed by the basal EEG/EMG recording, where Kcnn2 KO mice exhibited the significant decrease of NREM sleep (SWS) duration in addition to the significant decrease of total sleep duration (Figures 2G, 2H, and S2L). To further validate the role of Kcnn2 gene in the regulation of sleep duration, we conducted a sleep deprivation (SD) experiment to Kcnn2 KO mice and WT mice (Figure 2I). We compared NREM sleep (SWS) duration between the basal recording and the recovery period after 8 hr SD for Kcnn2 KO mice and WT mice, respectively. The WT mice exhibited a significant change in NREM sleep (SWS) duration (ZT12-ZT24) between the basal recording and the recovery period after 8 hr SD (ZT4-ZT12) (p < 0.01, Figure 2I). On the other hand, Kcnn2 KO mice exhibited no significant change in NREM sleep (SWS) duration (Figure 2I), indicating a change in the regulation of sleep duration in Kcnn2 KO mice. These results further support the hypothesis that the Ca²⁺-dependent hyperpolarization mechanism underlies the regulation of sleep duration in mammals.

We also noted that *Kcnma1* exhibited decreased sleep duration during morning and increased sleep duration during night (Figure S2I), which is consistent with the previously reported circadian phenotype of the KO animal for this gene (Meredith et al., 2006), further validating our methods for producing and phenotyping KO mice. Taken together, these results validated the first prediction of the AN model that an impairment of K_{Ca} channels would decrease sleep duration.

Impairment of Voltage-Gated Ca²⁺ Channels Decreases Sleep Duration

To further validate the AN model, we next tested the second prediction that the downregulation of Ca_v channels would decrease the sleep duration. The Ca_v channels are Ca²⁺ channels that open in response to membrane depolarization resulting in Ca²⁺ influx. They are usually composed of four or five distinct subunits (α_1 , α_2 , β , γ , and δ). Among them, the α_1 subunit mainly determines the channels' responsiveness and behavior in response to membrane depolarization. At least ten α_1 subunits are found in mammals, and they are divided into three well-defined and closely related groups by their sequence similarity (Catterall et al., 2005).

We created KOs of all of the ten Ca_v channel α_1 subunits (Cav1.1 [Cacna1s], Cav1.2 [Cacna1c], Cav1.3 [Cacna1d], Cav1.4 [Cacna1f], Cav2.1 [Cacna1a], Cav2.2 [Cacna1b], Cav2.3 [Cacna1e], Ca_v3.1 [Cacna1g], Ca_v3.2 [Cacna1h], and Ca_v3.3 [Cacna1i]) by injecting three gRNAs for each gene (Figures S4A-S4J and S4M-S4R; Tables S2 and S3). We confirmed the lethal phenotype seen in Cacna1s, Cacna1c, and Cacna1a biallelic KO mice generated by conventional methods (Austin et al., 2004; Jun et al., 1999; Seisenberger et al., 2000) also occurred in KO mice generated by the triple-target CRISPR method. We also observed the lethal phenotype in Cacna1d KO mice generated by the triple-target CRISPR method, which targeted all known isoforms of Cacna1d, including two isoforms that were not targeted in viable Cacna1d KO mice generated by conventional methods (Namkung et al., 2001; Platzer et al., 2000). As predicted from the AN model, Cacna1g, Cacna1h, and Cacna1f KO mice exhibited significant decrease in sleep duration (Figures 3A-3C). In particular, Cacna1g and Cacna1h KO mice showed strong short-sleeper phenotypes, with sleep duration of 655.1 \pm 13.4 min (mean \pm SEM, n = 10) and 647.6 \pm 20.1 min (mean \pm SEM, n = 5), respectively, which were 80.5 min (1.59 SD, p < 0.001) and 88.1 min (1.73 SD, p < 0.001) shorter than that of WT (Figure 3C). As further validation, we generated another group of Cacna1g and Cacna1h KO mice by independent CRISPR/Cas9 probe sets (set 2) and confirmed the observed short-sleeper phenotypes (Figures 3D-3F; Figures S4K, S4L, and S4S-S4U; Tables S2 and S3). These results strongly suggested that the short-sleeper phenotype of Cacna1g KO and Cacna1h KO mice cannot be attributed to the possible off-target effect of CRISPR but rather to the common genomic defects in Cacna1g and Cacna1h genes, respectively. The short-sleeper phenotype of Cacna1g KO mice in this study was consistent with the previous reports (Anderson et al., 2005; Lee et al., 2004). No significant change in sleep duration of Cacna1b KO mice was also consistent with the previous report (Beuckmann et al., 2003). Taken together, these results confirmed the second prediction that an impairment of Ca_v channels would decrease sleep duration.

Impairment of NMDA Receptors Decreases Sleep Duration

To further validate the AN model, we examined the third prediction that downregulation of NMDARs would decrease the sleep



Figure 3. Impairment of Voltage-Gated Ca²⁺ Channels Decreases Sleep Duration

(A) Sleep duration (per hour) over 6 days for KO mice of the $\mbox{Ca}_{\rm v}$ channel family.

(B) Sleep duration (per hour) over 24 hr, averaged over 6 days in KO mice of the Ca_v channel family. Red lines: the mean sleep duration at each time of day for each strain. Gray: WT (n = 108). Shaded area: SEM for each time point.

(C) Distributions of sleep/wake parameters of KO mice of the Ca_v channel family. Histogram: WT (n = 108). Black dashed line and gray shade: the mean and 1 SD range from the recording of WT.

(D–F) Sleep-duration phenotype for another group of Cacna1g and Cacna1h KO mice (set 2). Sleep duration (per hour) over 6 days (D), sleep duration (per hour) over 24 hr, averaged over 6 days (E), and distributions of sleep/wake parameters (F) are shown.

WT, C57BL/6N male mice. Error bars, SEM, *p < 0.05, **p < 0.01, ***p < 0.001, see also Supplemental Experimental Procedures for details.

duration. In the mouse genome, seven different subunits are categorized into the NMDAR family based on their sequence similarity. These NMDAR subunits form three well-defined and closely related subfamilies: the *GluN1* (*Nr1*) subunit, four distinct GluN2 subunits, *GluN2A* (*Nr2a*), *GluN2B* (*Nr2b*), *GluN2C* (*Nr2c*), and *GluN2D* (*Nr2d*), and two GluN3 subunits, *GluN3A* (*Nr3a*) and *GluN3B* (*Nr3b*). NMDARs function as a heterotetramer in which Nr1 subunits typically assemble with Nr2 subunits or a mixture of Nr2 and Nr3 subunits (Cull-Candy and Leszkiewicz, 2004; Paoletti, 2011; Traynelis et al., 2010). In contrast to the K_{Ca} channel family, KO mice of either *Nr1* or *Nr2b*, which are two main subunits of NMDAR family, exhibit lethal phenotype (Forrest et al., 1994; Li et al., 1994; Sunagawa et al., 2016). Therefore, we inhibited the NMDARs pharmacologically. First, the intraperitoneal (i.p.)



Figure 4. Impairment of NMDA Receptors Decreases Sleep Duration

(A and C) Dose-dependent sleep phenotype resulting from a single i.p. injection of phencyclidine (PCP) (A) and MK-801(C). Heatmaps: the sleep duration (per hour) for all mice. Red bar: the period used for comparing the parameters in (B) and (D), respectively.

(B and D) Both PCP (B) and MK-801 (D) reduced the sleep duration and P_{ws} in a dose-dependent manner for 5 hr (ZT2 to 6) after their i.p. injections. (E) Sleep phenotype resulting from a single i.p. injection of MK-801 by the EEG/EMG recording. (F) The sleep duration. NREM sleep (SWS) dura-

(r) The sleep duration, NREM sleep (SWS) duration, REM sleep duration, P_{ws} and P_{sw} for 5 hr (ZT2 to ZT6) after MK-801 i.p. injection by the EEG/ EMG recording.

Error bars, SEM, *p < 0.05, **p < 0.01, ***p < 0.01, ***p < 0.001, see also Supplemental Experimental Procedures for details.

would increase the excitability of cortical neurons, especially glutamatergic pyramidal neurons that we modeled in this study. This prediction is somewhat counter-intuitive, because NMDARs mediate "excitatory" positive-ion currents from outside to the inside of neurons, but it makes sense, given that the Ca²⁺-dependent hyperpolarization pathway is initiated by Ca²⁺ entry through NMDARs. To test this prediction, we performed a whole-brain imaging of neural activity at single-cell resolution using CUBIC analysis (Susaki et al., 2014) and Arc-dVenus mice, in which a destabilized

injection of phencyclidine (PCP), a well-known antagonist of NMDARs, into WT mice resulted in significantly decreased sleep duration as predicted (Figures 4A and 4B). We also confirmed this decreased sleep duration by a more specific NMDAR antagonist, MK-801 (Figures 4C and 4D) (Koek et al., 1988). The observed short-sleep phenotype was also confirmed by EEG/ EMG recording, where the MK-801-treated mice exhibited the significant decrease of NREM sleep (SWS) duration in addition to a significant decrease of total sleep duration (Figures 4E and 4F). We also note that this short-sleep phenotype was observed in the other strain of mice, C57BL/6J (Figures S5A-S5D). In addition, we chronically administered MK-801 to C57BL/6J mice (the drug was diluted into sterile water to be 16, 32, 64, and 96 mg/L and administered as drinking water) and confirmed the decreased sleep-duration phenotype (Figures S5E-S5G). Taken together, these results confirmed our prediction that impaired NMDAR function would decrease sleep duration.

Impairment of the Ca²⁺-Dependent Hyperpolarization Pathway Increases Neural Excitation

By using the chronic pharmacological impairment of NMDARs, we also tested our prediction that this impairment of NMDARs

version of yellow fluorescence protein Venus is expressed upon neural excitation, under control of Arc gene promoter. To minimize temporal variations, we sampled Arc-dVenus mice every 6 hr for 1 day under constant darkness, with or without the chronic administration of NMDAR antagonist, MK-801. We noticed a temporal variation of Venus signals in the Arc-dVenus mice without drug administration (Figure S6A; Movie S1). Venus signals were relatively low during the subjective day (circadian time [CT] 4 and 10), when mice are relatively inactive, and were relatively high during the subjective night (CT16 and CT22), a higher activity time for mice (Figure S6B). In contrast, in the MK-801-treated Arc-dVenus mice, the Venus signals exhibited different temporal patterns (Figure S6A; Movie S2). Importantly, these altered Venus expression patterns matched the altered behavioral patterns of the MK-801-treated mice (Figure S6B). Notably, close inspection of whole-brain single-cell resolution imaging data revealed that some cells were highly activated with high-intensity Venus signals both in MK-801-treated and untreated mice (Figure S6C). Importantly, the MK-801-treated mice exhibited significantly more hyper-activated cells than the untreated mice did (Figure 5A). We also noticed that the total number of hyper-activated cells was well correlated with the total



intensity of hyper-activated cells (Figure S6D), which allowed us to perform the simplified computational analysis and obtained spatio-temporal profiling of cellular activities for different regions over the entire brain (Figure 5A). Subsequent hierarchical clustering analysis (Figure 5B; Table S6) revealed that individual brain regions follow a few common patterns of cellular activities (Figures 5C–5E; Figure S6E; Movie S3). For example, brain regions in the pink cluster such as various regions of cerebral cortex exhibit higher intensity in subjective day (CT4 or CT10) in the MK-801-treated mice, whereas they exhibit comparable intensity in subjective night (CT16 or CT22) (Figures 5C and 5F). Brain regions in the yellow cluster such as cerebellar cortex exhibit much higher intensity in early subjective day (CT4) in the MK-801-treated mice, whereas they exhibit comparable intensity in use higher intensity in early subjective day (CT4) in the MK-801-treated mice, whereas they exhibit comparable intensity in use higher intensity in early subjective day (CT4) in the MK-801-treated mice, whereas they exhibit comparable intensity in use the performance of the figure set of the figure se

Figure 5. Impairment of the Ca²⁺-Dependent Hyperpolarization Pathway Increases Neural Excitation

(A) Temporal variation of the number of hyperactivated cells in the whole brain. Left: representative 3D-reconstituted brain images of the Arc-dVenus signals (green) from hyper-activated cells shown for mice with or without the chronic administration of MK-801. Gray, TOPRO3 signals. Scale bar, 2,000 μ m. Right: the number of hyperactivated cells in the whole brain. CT, Circadian time.

(B) Heatmap of the brain regions, clustered based on the normalized intensity across the four time points.

(C–E) Temporal variation of the number of hyperactivated cells in specific clusters. Left: 3Dreconstituted brain images of the Arc-dVenus signals from hyper-activated cells that located in regions belonging to the same heatmap cluster (shown in pink in C, yellow in D, and blue in E). Gray, TOPRO3. Scale bar, 2,000 μ m. Right: estimated number of hyper-activated cells.

(F) Hierarchical representation of the Allen Brain Atlas annotation. Nodes represent brain regions and are colored based on the cluster they belong in the heatmap. Light gray: low-signal regions excluded from the clustering. CH, cerebrum; BS, brain stem; CB, cerebellum. Error bars, SEM.

other time points (CT10, CT16, or CT22) (Figures 5D and 5F). In contrast, brain regions in the blue cluster such as hippocampus exhibit higher intensity at certain time points in the untreated mice, whereas they exhibit relatively low intensity in MK-801-treated mice (Figures 5E and 5F).

In this study, we modeled glutamatergic pyramidal neurons in the layers 4-6. To stringently validate the prediction from the AN model, we next performed temporal profiling of cellular activities for different layers in the cerebral cortex. We found each layer exhibits higher in-

tensity in subjective day (CT4 or CT10) in the MK-801-treated mice than in the untreated mice, whereas they exhibit comparable intensity in subjective night (CT16 or CT22) (Figures 6A–6C; Movie S4). In particular, the layer 4-6 exhibited significant (p < 0.05) and strongest (~400 hyper-activated cells/mm³) response to the MK-801 injection (Figures 6C and 6D, right). To determine the cellular identify of hyper-activated cells in the layer 4-6 of the MK-801-treated mice, we conducted fluorescence in situ hybridization (FISH) analysis of WT mice intraperitoneally injected with or without MK-801 and performed double-staining of *c-Fos* and *Nr2b* (Figures S6F and S6G; Figures 6E and 6F; Table S5), *Vglut1/2* (glutamatergic cell makers, Figure S6H; Figures 6H and 6J; Table S5), respectively. As a result, we found



Figure 6. Impairment of the Ca²⁺-Dependent Hyperpolarization Pathway Increases the Excitability of Glutamatergic Pyramidal Neurons (A–D) 3D-reconstituted brain images of the Arc-dVenus signals from hyper-activated cells belonging to the layer 1 (A, cyan), layer 2 and 3 (B, magenta), and layer

4, 5, and 6 (C, yellow). Gray, TOPRO3 signals. Scale bar, 2,000 μm. (D) Estimated number of hyper-activated cells.

(E and F) Images of FISH analysis using *c-Fos* and *Nr2b* probes of the cerebral cortex from mice subjected to i.p. saline (E) or MK-801 (F) injection. Scale bar, 100 μm.

(G) The activated cells expressing c-Fos mRNA in layer 4, 5, and 6 of c-Fos and Nr2b double-stained sections (E and F) were counted.

(H and I) Images of FISH analysis of layer 4, 5, and 6 from MK-801-treated mice using *c-Fos* and either *Vglut1/2* (H) or *Gad1/2* (I) probes, respectively. White arrowheads, *Vglut1/2*-negative cell. White arrows, *Gad1/2*-positive cell. Scale bar, 100 μm.

(J) The active cells responded to MK-801-injection in layer 4, 5, and 6 of *c-Fos* and *Vglut1/2* or *Gad1/2* double-stained sections (H and I) were counted. Error bars, SEM.

that MK-801-treated mice exhibited significantly more c-Fos-expressing cells in the layer 4-6 than the untreated mice did (Figures 6E–6G, p < 0.05). Importantly, almost all c-Fos-expressing cells in the layer 4-6 also expressed Nr2b mRNA (Figure 6G, p < 0.05), strongly suggesting that pharmacological inhibition of NR2B-containing NMDARs increased not indirectly but rather directly the excitability of the neuron. The Ca2+-dependent hyperpolarization hypothesis predicts that the inhibition of Ca²⁺ entry will induce the excitability of both glutamatergic and GABAergic neurons through the inhibition of Ca²⁺-dependent hyperpolarization pathway. Indeed, both numbers of glutamatergic and GABAergic neurons that express c-Fos were increased in MK-801-treated mice compared to untreated mice (Figures 6G and 6J), which was consistent with the direct excitation of these neurons by inhibiting NMDARs. We also note that most of the c-Fos-expressing cells in the layer 4-6 were glutamatergic cells (Figures 6H-6J). Taken together, these results confirmed the fourth prediction that impaired NMDAR functioning directly increases the excitability of neurons, especially glutamatergic pyramidal neurons that are modeled in this study.

Impairment of Plasma Membrane Ca²⁺ ATPase Increases Sleep Duration

To further validate the AN model, we next tested the last prediction that downregulation of Ca^{2+} pump would increase the sleep duration. The plasma membrane Ca^{2+} ATPases (PMCAs), a major member of Ca^{2+} pump, are transport proteins located in the plasma membrane of cells. PMCAs, whose function is powered by hydrolysis of ATP, pump Ca^{2+} out of cells.

We created KOs of all of the four PMCAs (PMCA1 [Atp2b1], PMCA2 [Atp2b2], PMCA3 [Atp2b3], and PMCA4 [Atp2b4]) by injecting three gRNAs for each gene (Figures S7A-S7D, S7J, S7K, and S7N; Tables S2 and S3). We confirmed the lethal phenotype seen in Atp2b1 biallelic KO mice generated by conventional methods (Okunade et al., 2004) also occurred in KO mice generated by the triple-target CRISPR method. As predicted from the AN model, Atp2b3 KO mice exhibited a significant increase in sleep duration with 777.8 ± 8.5 min (mean ± SEM, n = 12) in one day, which was 53.6 min (1.07 SD, p < 0.05) longer than that of WT mice (Figures 7A-7C). To confirm the significance of the observed long-sleeper phenotype, we generated another batch of Atp2b3 KO mice by the same CRISPR/Cas9 probe set (set 1 in UT, Figure S7L) and confirmed the reproducibility of the observed long-sleeper phenotype (p < 0.01, Figures 7D-7F). As further validation, we generated another group of Atp2b3 KO mice by independent CRISPR/Cas9 probe set (set 2, Figures S7I and S7M) and confirmed the significant long-sleeper phenotypes (p < 0.01, Figures 7D–7F; Tables S2 and S3). These results strongly suggested that the observed long-sleeper phenotype of Atp2b3 KO mice cannot be attributed to the possible off-target effect of CRISPR, but rather to the common genomic defects in Atp2b3 gene. Taken together, these results confirmed the last prediction that an impairment of Ca²⁺ pumps would increase sleep duration.

Impairment of Calcium/Calmodulin-Dependent Protein Kinase Decreases Sleep Duration

In this study, our average-neuron model and its experimental validation suggested that the molecular components in the

Ca²⁺-dependent hyperpolarization pathway can be potential molecular targets of homeostatic process S. In other words, our model supposed that the underlying molecular mechanism for homeostatic process S should target on the Ca²⁺-dependent hyperpolarization pathway. Such molecular mechanism is also supposed to be able to sense the awake and/or slow-wavesleep states (e.g., through the concentration or temporal patterns of Ca2+) to allow the reversible transitions between SWS and awake states. Although there are many intracellular molecules meeting these criteria for the underlying molecular mechanisms for homeostatic process S, we focused, as a first step, on the major protein kinase in the brain, calcium/calmodulin-dependent protein kinase type II (CaMKII), which is known to be able to interact with some of the Ca2+-dependent hyperpolarization pathway (i.e., CaMKII is also a well-known protein family, which can bind to NMDARs or L-type Ca²⁺ channels in response to Ca²⁺ influx) (Leonard et al., 1999; Rose et al., 2009). CaMKII is formed by 12 catalytically active subunits consisted of four different subunits (CaMKIIa [Camk2a], CaMKIIB [Camk2b], CaMKIIy [Camk2g], and CaMKIIS [Camk2d]) and their kinase activities are Ca²⁺ dependent. We thus created KOs of all of the four CaMKIIs by injecting three gRNAs for each gene (Figures S7E-S7H, S7P, S7Q, S7S, S7U, and S7V; Tables S2 and S3). Camk2a KO and Camk2b KO mice exhibited a significant decrease in sleep duration with 648.8 \pm 29.5 min (mean \pm SEM, n = 6) and 578.6 ± 14.7 min (mean \pm SEM. n = 5) in 1 day. respectively. which was 50.1 min (1.27 SD, p < 0.01) and 120.3 min (3.04 SD, p < 0.001) shorter than that of WT mice (Figures 7G–7I). As further validation, we generated another group of Camk2a KO mice and Camk2b KO mice by an independent CRISPR/Cas9 probe set (set 2, Figures S7O, S7R, and S7T; Tables S2 and S3) and confirmed the observed short-sleeper phenotypes (Figures 7J–7L). These results strongly suggested that the observed short-sleeper phenotype of Camk2a KO and Camk2b KO mice cannot be attributed to the possible off-target effect of CRISPR, but rather to the common genomic defects in Camk2a and Camk2b gene, respectively. Taken together, these results further support a hypothesis that Ca2+-dependent hyperpolarization pathway underlies the regulation of sleep duration in mammals.

DISCUSSION

Ca²⁺-Dependent Hyperpolarization Pathway as a Possible Molecular Target for Process S

In this study, we developed the AN model, which revealed that the molecular components of the Ca²⁺-dependent hyperpolarization pathway are potential molecular targets of the homeostatic sleep regulators (Figure 7M). This model suggests that assembled neurons themselves could have at least two default states, SWS and awake. It is consistent with the previous in vitro cortical model that showed transition from sleep-like state to wake-like state by stimulation with mixture of neurotransmitters (Hinard et al., 2012). In particular, the Ca²⁺-dependent mechanism of SWS firing patterns is consistent with the previous computational studies in electric bursting of various types of cells (Arbib, 2003; Izhikevich, 2007). How the molecules, such as K_{Ca} channels *Kcnn2* and *Kcnn3*, the Ca_v channels *Cacna1g* and *Cacna1h*, and MK-801-targeted NMDAR subunits (possibly *Nr1/Nr2b*)



Figure 7. Impairment of Ca²⁺ Pumps Increases Sleep Duration and Impairment of CaMKIIs Decreases Sleep Duration (A) Sleep duration (per hour) over 6 days for KO mice of the PMCA family.

(B) Sleep duration (per hour) over 24 hr, averaged over 6 days in KO mice of the PMCA family. Red lines, the mean sleep duration at each time of day for each strain. Gray, WT (n = 108). Shaded area, SEM for each time point.

(C) Distributions of sleep/wake parameters of KO mice of the plasma membrane Ca²⁺ ATPase family. Histogram, WT (n = 108). Black dashed line and gray shade, the mean and 1 SD range from the recording of WT.

(D–F) Sleep-duration phenotype for another batch (set 1) and another group (set 2) of *Atp2b3* KO mice. Sleep duration (per hour) over 6 days (D), sleep duration (per hour) over 24 hr, averaged over 6 days (E), and distributions of sleep/wake parameters (F) are shown.

(G) Sleep duration (per hour) over 6 days for KO mice of the CaMKII family by triple-target CRISPR.

(legend continued on next page)

and the Ca²⁺ pumps Atp2b3 are targeted by the sleep homeostatic process S remains to be solved. One possibility is that process S may be represented as the amount of SISs, which then regulate these molecules. In this context, it will be interesting to examine the relationship of SISs (e.g., adenosine, NO, prostaglandin D, TNF, IL1, and GHRH) (Clinton et al., 2011; Krueger et al., 2008; Obal and Krueger, 2003) to the molecules involved in the Ca²⁺-dependent hyperpolarization pathway. Alternatively, but not exclusively, process S may be represented directly as the quality or quantity of the molecules involved in the Ca²⁺-dependent hyperpolarization pathway. In that case, process S can be identified as an activity-dependent effect (e.g., phosphorylation) and/or a mechanism affecting the quantity (e.g., translation) of these target molecules. Given the increasing evidence for the post-translational modification of K_{Ca} channels, Ca_v channels, NMDARs, and Ca²⁺ pumps, one of the plausible execution mechanisms of homeostatic process S might be activity-dependent protein modifications (e.g., phosphorylation or dephosphorylation) of the Ca²⁺-dependent hyperpolarization pathway. The evidence that impairment of Camk2a and Camk2b, a well-known bounder and modifier of Cav channels and NMDARs, decreased sleep duration may support this mechanism. Interestingly, since protein modifications are often involved in sleep-related neurological processes that occur over long timescales (e.g., hours to days), such as learning, memory, and circadian clocks, a role for such activity-dependent protein modifications in the Ca²⁺dependent hyperpolarization pathway is compatible with the slow and macroscopic dynamics of sleep. Compared to the homeostatic (i.e., feedback) control expected for process S, circadian (i.e., feedforward) control of the guality and/or guantity of the molecules involved in the Ca2+-dependent hyperpolarization pathway might be also important especially for process C of sleep (Borbély, 1982). Recently, a dopamine synthesis pathway was reported to be regulated by circadian clock in mammals (Chung et al., 2014). In this context, it will be interesting to examine the relationship of awake-inducing substances (e.g., dopamine) to the molecules involved in the Ca2+-dependent hyperpolarization pathway. We also noted previous reports that process C (circadian clock) is regulated by calcium-dependent pathways such as rhyanodine receptors, calcium-dependent potassium channels, voltage-gated calcium channels, and calcium binding proteins (Ding et al., 1998; Ikeda et al., 2003; Meredith et al., 2006; Schmutz et al., 2014; Stadler et al., 2010). In either case, gradual changes in the quality and/or quantity of such target molecules in the Ca2+-dependent hyperpolarization pathway, at least in theory, could eventually alter the fast/microscopic electrophysiological patterns of neurons, suggesting that this mechanism is also compatible both with slow/macroscopic homeostatic regulations and fast/microscopic electro-physiology. Therefore, the Ca²⁺-dependent hyperpolarization hypothesis has the potential to connect the gaps between slow/ macroscopic homeostatic regulations and fast/microscopic electrophysiological dynamics.

A Possible Role of Ca²⁺-Dependent Hyperpolarization Pathway in Slow-Wave Sleep and Sleep Homeostasis

In this study, we performed mammalian reverse genetics without crossing by combining the recently developed triple-target CRISPR method and the non-invasive sleep/wake recording system SSS, which allowed us to comprehensively generate more than 21 KO mice and efficiently analyze their sleep/wake phenotype. As a result, seven KO mice, including Kcnn2, Kcnn3, Cacna1g, Cacna1h, Atp2b3, Camk2a, and Camk2b KO mice, exhibited the significant change in sleep duration. These sleep/ wake phenotypes were confirmed by another group of corresponding KO mice by independent CRISPR/Cas9 probe set (set 2). We also noted that pharmacological inhibition of NMDARs by MK-801 reduced sleep duration. Although there is the possibility that these gene knockouts might affect the phenotype of respiration and SSS system could miscalculate their sleep duration, the EEG/EMG recording of Kcnn2 KO mice and MK-801-treated mice confirmed these mice exhibited the significant decrease of NREM sleep (SWS) duration in addition to the significant decrease of total sleep duration. These results supported the role of Ca²⁺dependent hyperpolarization pathway in the regulation of sleep duration. In addition, these EEG/EMG results also suggest that the observed decrease of sleep duration was mainly due to the decrease of NREM sleep (SWS) duration at least in Kcnn2 KO mice and MK-801-treated mice. Further EEG/EMG recording of the remaining six KO mice will clarify the relationship between Ca²⁺-dependent hyperpolarization pathway and SWS.

Another interesting question is about the relationship between Ca^{2+} -dependent hyperpolarization pathway and sleep homeostasis. As the very first step, we conducted an SD experiment to *Kcnn2* KO mice and WT mice, which revealed that WT mice exhibited significant change in NREM sleep (SWS) duration after SD. On the other hand, *Kcnn2* KO mice exhibited no significant change in NREM sleep (SWS) duration, implying a possible role of *Kcnn2* in sleep homeostasis. Further experimental validations (e.g., the SD experiments of the remaining six KO mice and MK-801-treated mice) will also clarify the relationship between Ca²⁺-dependent hyperpolarization pathway and sleep homeostasis.

A Systems Biology Approach to Examine Sleep/Wake Cycles

Although the molecular identity of the sleep homeostatic process S is still unknown, our findings predict that process S can be implemented as a neural-activity-dependent quality

⁽H) Sleep duration (per hour) over 24 hr, averaged over 6 days in KO mice of the CaMKII family. Red lines, the mean sleep duration at each time of day for each strain. Gray, WT (n = 101). Shaded area, SEM for each time point.

⁽I) Distributions of sleep/wake parameters of KO mice of the CaMKII family. Histogram, WT (n = 101). Black dashed line and gray shade, the mean and 1 SD range from the recording of WT.

⁽J–L) Sleep-duration phenotype for another group of Camk2a and Camk2b KO mice (set 2). Sleep duration (per hour) over 6 days (J) sleep duration (per hour) over 24 hr, averaged over 6 days (K), and distributions of sleep/wake parameters (L) are shown.

⁽M) A model diagram of Ca²⁺-dependent hyperpolarization pathway.

WT, C57BL/6N male mice. Error bars, SEM, *p < 0.05, **p < 0.01, ***p < 0.001, see also Supplemental Experimental Procedures for details.

and/or quantity control of the Ca²⁺-dependent hyperpolarization pathway. Therefore, the potential molecular targets for process S (e.g., *Kcnn2*, *Kcnn3*, *Cacna1g*, *Cacna1h*, *Nr1/Nr2b*, and *Atp2b3*) can be used to identify the missing switch (i.e., process S) between sleep/wake cycles. We also note that *Camk2a* and *Camk2b* are one of the candidate molecules underlying homeostatic process S. In this study, we focused on the production and phenotype-analysis of global knockout mice. The obvious next step will be the cell-type-specific analysis of these regulators of sleep duration found in this study (Susaki and Ueda, 2016). This new research field, i.e., the system-level identification and analysis of the molecular networks underlying sleep/ wake cycles in mammals, will expand our detailed understanding of sleep regulation, sleep disorders, and its associated diseases such as psychiatric diseases in the years to come.

EXPERIMENTAL PROCEDURES

Details are also supplied in the Supplemental Experimental Procedures.

Simulations

The AN model was constructed by simplifying previously published Hodgkin-Huxley-type models of the electrophysiology of cortical neuron network into the AN. The full system of equations is presented in Supplemental Experimental Procedures. The parameter values are shown in Table S1. Parameter searches for SWS firing patterns were conducted in the defined parameter space including biophysically reasonable parameter values. In the bifurcation analysis, each parameter value was gradually varied from the parameter sets that give SWS firing patterns obtained from parameter searches. Transitions from SWS firing patterns to awake firing patterns were then counted.

Animals and Sleep Phenotyping

All experimental procedures and housing conditions involving animals and their care were approved by the Animal Care and the Use Committee. Sleep phenotyping was done non-invasively by SSS. Creation and sleep phenotyping of several KO mice were done in University of Tokyo by using C57BL/6N mice (n = 101) as control information (Figures 3D–3F; Figures 7J–7M). All other experiments were done in RIKEN by using C57BL/6N mice (n = 108) published in our previous paper as control information (Sunagawa et al., 2016). The basal EEG/ EMG recording for *Kcnn2* KO mice was performed in University of Tokyo. Sleep deprivation experiment using gentle handling of *Kcnn2* KO mice was conducted after the basal EEG/EMG recording. CRISPR-KO animals were generated by one-cell embryo microinjection of synthesized *Cas9* mRNA and gRNAs (mix of three targeting sequences; template sequences are shown in Table S2) to C57BL/6N fertilized eggs. KO genotype was confirmed by qPCR and/or genomic sequencing. We also used Arc-dVenus Tg mice (line D) (Eguchi and Ya-maguchi, 2009) for MK-801 administration experiments and CUBIC analysis.

Pharmacological Administration

C57BL/6N or C57BL/6J mice received an i.p. injection of synthesized PCP or MK-801 maleate in each concentration (Figures 4A–4D; Figures S5A–S5D) at ZT2, respectively. For chronic administration of MK-801, the drug was administered to C57BL/6J mice and Arc-dVenus mice as drinking water in each concentration. For EEG/EMG recording and FISH, 2 mg/kg of MK-801 was injected i.p. to C57BL/6N mice at ZT2.

CUBIC Analysis of Arc-dVenus Tg Animals

Fixed brains of Arc-dVenus mice were cleared and their whole brain images were acquired and subjected to generate 3D NIfTI-1 files as previously reported (Susaki et al., 2014, 2015), with some modifications. The Arc-dVenus signals were normalized and used for comparison. The hyper-activated cells were detected using the Fiji software (Schindelin et al., 2012) or analyzed by creating binary mask for each anatomical region and applied it to the reconstructed 3D images showing only hyper-activated cells. Querying specific

regions relies on images aligned to the Allen Brain Atlas (Lein et al., 2007). The intensity within each region can be also used to estimate the number of hyper-activated cells. Clustering and hierarchical annotation of anatomical regions were performed to the dataset (Figure 5; Figures 6A–6D).

Fluorescence In Situ Hybridization

The FISH protocol was based on TAI-FISH (Xiu et al., 2014) with some modifications. The RNA probes for hybridization (PCR primers for probe templates are shown in Table S5) labeled with digoxigenin (DIG)-UTP or fluorescein (Flu)-UTP and detected as cyanine 3 or Alexa Fluor 488 signals, respectively. Signals were amplified with Tyramide Signal Amplification system (PerkinElmer). Images of these sections were acquired with an inverted confocal microscope (FV1200/IX83) and an upright fluorescent microscope (BX51, Olympus) and were analyzed with ImageJ (Preibisch et al., 2009).

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, seven figures, six tables, and four movies and can be found with this article online at http://dx.doi.org/10.1016/j.neuron.2016.02.032.

A video abstract is available at http://dx.doi.org/10.1016/j.neuron.2016.02. 032#mmc10.

AUTHOR CONTRIBUTIONS

H.R.U., F.T., G.A.S., and S.S. designed the study. F.T. constructed and analyzed the averaged-neuron model. K.S., M.U.-T., R.O., and S.S. performed the triple-target CRISPR. G.A.S., D.T., K.L.O., and S.S. performed the sleep phenotype analysis. D.T. performed the sleep deprivation experiment. H.Y. and E.A.S. performed CUBIC treatment of mice brains. K.M. synthesized PCP. D.P. and H.Y. developed CUBIC informatics on spatio-temporal profiling of cellular activities of individual regions over the entire brain. E.A.S. and S.S. performed in situ hybridization. M.U.-T. and H.F. performed the genotyping of animals. H.R.U., F.T., G.A.S., and S.S. wrote the manuscript. All authors discussed the results and commented on the manuscript text.

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Supplemental Information

Involvement of Ca²⁺-Dependent Hyperpolarization

in Sleep Duration in Mammals

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1 Supplemental Experimental Procedures

2 Averaged-neuron model

To recapitulate the electrophysiological characteristics of the slow-wave-sleep and
awake states, a computational model was constructed as follows:

$$CA\frac{dV}{dt} = -A(I_{L} + I_{Na} + I_{K} + I_{A} + I_{KS} + I_{Ca} + I_{KCa} + I_{NaP} + I_{AR})$$
$$-I_{NMDA} - I_{AMPA} - I_{GABA}$$

5 , where C is the membrane capacitance, A is the area of a single neuron, V is the 6 membrane potential, and I denotes the current of the indicated ion channels. Please 7 note that both sides except extrinsic (synaptic) ion currents should be multiplied by 10 8 to adjust their units to nA if the numerical values listed in Table S1 are directly used in 9 the numerical simulation. The intrinsic (non-synaptic) ion channels in the soma were 10 modeled based on previous studies (Bazhenov et al., 2002; Chen et al., 2012; 11 Compte et al., 2003; Hill and Tononi, 2005; Sanchez-Vives et al., 2010; Timofeev 12 et al., 2000) using Hodgkin-Huxley-type equations, with a gating variable x governed 13 by the first-order kinetics equation:

$$\frac{dx}{dt} = \phi[\alpha_x(V)(1-x) - \beta_x(V)x] = \frac{\phi\left[x_{\infty}(V) - x\right]}{\tau_x(V)}$$

14 , where ϕ is the temperature factor (ϕ = 1). Each intrinsic current is given by the 15 following equations (1) to (9):

$$I_L = g_L (V - V_L) \tag{1}$$

$$I_{Na} = g_{Na} m_{Na\infty}^{3} h_{Na} (V - V_{Na})$$

$$m_{Na\infty} = \alpha_m / (\alpha_m + \beta_m)$$

$$\alpha_m = 0.1(V + 33) / [1 - exp(-(V + 33)/10)]$$

$$\beta_m = 4exp(-(V + 53.7)/12)$$

$$\frac{dh_{na}}{dt} = 4(\alpha_h (1 - h_{Na}) - \beta_h h_{Na})$$

$$\alpha_h = 0.07exp(-(V + 50)/10)$$

$$\beta_h = 1 / [1 + exp(-(V + 20)/10)]$$

$$I_K = g_K n_K^4 (V - V_K)$$

$$\frac{dn_k}{dt} = 4(\alpha_n (1 - n_K) - \beta_n n_K)$$

$$\alpha_n = 0.01(V + 34) / [1 - exp(-(V + 34)/10)]$$
(2)

 α_m

$$\beta_n = 0.125 \exp(-(V + 44)/25)$$

$$I_A = g_A m_{A\infty}^3 h_A (V - V_K)$$
(4)

 $m_{A\infty} = 1/[1 + \exp(-(V + 50)/20]$ $\frac{dh_A}{dt} = \left(h_{A\infty} - h_A\right) / \tau_{hA}$ $h_{A\infty} = 1/[1 + \exp((V + 80)/6)]$ $I_{KS} = g_{KS} m_{KS} (V - V_K)$

(5)

$$\frac{dm_{KS}}{dt} = (m_{KS\infty} - m_{KS})/\tau_{mKS}$$
$$m_{KS\infty} = 1/[1 + \exp(-(V + 34)/6.5)]$$

 $\tau_{mKS} = 8/[\exp(-(V+55)/30) + \exp((V+55)/30)]$

$$I_{Ca} = g_{Ca} m_{Ca\infty}^2 (V - V_{Ca})$$
(6)

$$m_{Ca\,\infty} = 1/[1 + exp(-(V + 20)/9)]$$
$$I_{KCa} = g_{KCa}m_{KCa\,\infty}(V - V_K)$$
(7)

 $m_{KCa\infty} = 1/[1 + (K_D/[Ca^{2+}])^{3.5}]$

$$I_{NaP} = g_{NaP} m_{NaP\,\infty}^3 (V - V_{Na}) \tag{8}$$

$$m_{NaP\infty} = 1/[1 + \exp(-(V + 55.7)/7.7)]$$

$$I_{AR} = g_{AR} h_{AR\,\infty} (V - V_K) \tag{9}$$

$$h_{AR\infty} = 1/[1 + \exp((V + 75)/4)]$$

The extrinsic (synaptic) ion currents in the dendrite were also modeled based
on previous studies (Bazhenov et al., 2002; Chen et al., 2012; Compte et al., 2003;
Hill and Tononi, 2005; Sanchez-Vives et al., 2010; Timofeev et al., 2000) using
Hodgkin-Huxley-type equations, with a gating variable *s* governed by the first-order
kinetics equation for AMPA and GABA receptors:

$$\frac{ds}{dt} = \alpha f(V) - \frac{s}{\tau}$$

- 1 , and with gating variables s and x governed by the second-order kinetics equations
- 2 for NMDA receptors:

$$\frac{ds}{dt} = \alpha_s x(1-s) - \frac{s}{\tau_s}$$
$$\frac{dx}{dt} = \alpha_x f(V) - \frac{x}{\tau_x}$$

- 3 , where τ is the time constant for turnover of s and x, V is the membrane potential, and
- 4 *f* is a saturating function of *V*:

$$f(V) = 1/[1 + \exp(-(V - 20)/2)]$$

5 Each extrinsic current is then given by the following equations (10) to (12):

$$I_{AMPA} = g_{AMPA} s_{AMPA} (V - V_{AMPA})$$
(10)
$$\frac{ds_{AMPA}}{dt} = 3.48f(V) - \frac{s_{AMPA}}{\tau_{AMPA}}$$

$$I_{NMDA} = g_{NMDA} s_{NMDA} (V - V_{NMDA})$$
(11)

$$\frac{ds_{NMDA}}{dt} = 0.5x_{NMDA}(1 - s_{NMDA}) - \frac{s_{NMDA}}{\tau_{sNMDA}}$$
$$\frac{dx_{NMDA}}{dt} = 3.48f(V) - \frac{x_{NMDA}}{\tau_{xNMDA}}$$
$$I_{GABA} = g_{GABA}s_{GABA}(V - V_{GABA})$$
$$\frac{ds_{GABA}}{dt} = f(V) - \frac{s_{GABA}}{\tau_{GABA}}$$
(12)

6

The Ca²⁺ concentration was also modeled by the following equation (13):

$$\frac{d[Ca^{2+}]}{dt} = -\alpha_{Ca}(AI_{Ca} + I_{NMDA}) - \frac{[Ca^{2+}]}{\tau_{Ca}}$$
(13)

Please note that intrinsic (non-synaptic) ion currents (i.e. *I_{Ca}*) should be
 multiplied by 10 to adjust its unit to nA if the numerical values listed in **Table S1** are
 directly used in the numerical simulation.

4 The parameters values were based on previous studies (Bazhenov et al., 5 2002; Chen et al., 2012; Compte et al., 2003; Hill and Tononi, 2005; 6 Sanchez-Vives et al., 2010; Timofeev et al., 2000) with some modifications, and 7 listed in **Table S1**. The model was simulated using Mathematica (version 9.0, 8 Wolfram Research, Inc.), with the NDSolve function, in which MaxSteps and 9 PrecisionGoal were chosen to be infinite and 15, respectively. Integration was 10 performed with the following initial values in each simulation, parameter search, and 11 bifurcation analysis: V = -45 mV, $h_{Na} = 0.045$ (unitless), $n_{K} = 0.54$ (unitless), $h_{A} =$ 0.045 (unitless), $m_{\rm KS} = 0.34$ (unitless), $[Ca^{2+}] = 1 \,\mu M$, $s_{\rm AMPA} = 0.01$ (unitless), $s_{\rm NMDA} =$ 12 0.01 (unitless), $x_{\text{NMDA}} = 0.01$ (unitless), $s_{\text{GABA}} = 0.01$ (unitless). 13

14

15 Parameter search

16 We computationally searched for the parameter sets that yield slow-wave-sleep firing 17 patterns by numerically calculating the model's behavior using parameter sets randomly chosen from a large parameter space. The parameter space was defined to 18 19 include biophysically reasonable parameter values: the conductance of intrinsic (non-synaptic) channels in the soma (g_L , g_{Na} , g_K , g_A , g_{KS} , g_{Ca} , g_{KCa} , g_{NaP} , g_{AR}) and 20 extrinsic (synaptic) channels in the dendrite (g_{AMPA} , g_{NMDA} , g_{GABA}) were generated by 21 22 selecting parameters from an exponential distribution bounded to the interval 0.01-100 mS/cm² and 0.002-20 µS, respectively. The intervals used in the parameter 23

searches for the conductance of the intrinsic and extrinsic channels were set to be the same range after the consideration of the area of a neuron, $A = 0.02 \text{ mm}^2$ (i.e.

3 $0.002-20 \ \mu\text{S}/ 0.02 \ \text{mm}^2 = 0.01-100 \ \text{mS/cm}^2$). We also conducted a random parameter 4 search for τ_{Ca} (10-1000 ms). These parameters were chosen because they could be 5 perturbed by genetic/pharmacological manipulations, and we can predict the outcome 6 of such perturbation based on our parameter search.

7 We analyzed the parameters that yielded a steady-state or periodic solution 8 with real values under the defined computer memory usage and calculation time. We 9 note that most cases exhibited steady-state or periodic behavior. The major 10 frequency of the oscillatory behavior was then analyzed by fast Fourier transform. We 11 also evaluated the fine structure of the oscillatory behavior by counting the number of 12 spikes in 1 second; the number of spikes was determined as half the number of times 13 the membrane potential crossed -20 mV. Solutions, in which the membrane potential 14 exceeded this threshold at almost all time points (> 95%), were eliminated at this 15 point. Based on these characteristics, the solutions were classified into four 16 categories: "Resting" (spike numbers per second < 2 or peak frequency = 0 Hz), 17 "Sleep" (0 Hz < peak frequency < 10 Hz and spike number per second > 5 × peak frequency), "Wake" (peak frequency \geq 10 Hz), and "Slow-wave with few spikes" (0 Hz) 18 19 < peak frequency <10 Hz and spike number per second < 5 × peak frequency). 20 "Slow-wave with few spikes" indicated that the solution represented slow-wave 21 activity, with fewer than five spikes during one bursting phase of neural activity. All of 22 the solutions classified as "Sleep" were then checked manually to select the ones that 23 exhibit oscillatory membrane potential alternating between bursting and silent 24 phases.

1 Bifurcation analysis

2 To analyze the behavior of the system around the parameter set that gave 3 slow-wave-sleep firing patterns, we conducted two types (i.e. comprehensive and 4 simplified versions) of bifurcation analysis. In the comprehensive bifurcation analysis 5 (Figures 1C and 1F), each conductance or the time constant was gradually changed 6 one by one from 0.001 to 10 times its original value, and the solutions were 7 automatically classified into four categories according to the criteria described above. 8 When the channel conductance of excitatory synaptic currents (g_{AMPA} , g_{NMDA}) 9 decreased, the solution sometimes showed a cyclic firing pattern (peak frequency > 10 10 Hz) but weak synaptic currents. In these cases, we classified the behavior as 11 "Wake" if either of the two excitatory synaptic currents (I_{NMDA} , I_{AMPA}) was > 50% of that 12 of the original parameter set. Otherwise, the solutions were classified as "Cyclic firing" 13 with weak synaptic currents" (Figure 1F).

14 In the simplified bifurcation analysis (Figures 1G-1I; Figure S1K), each 15 conductance or time constant was changed to have 0.001 times its original value. The 16 solution was classified into five categories as described above. When the parameters 17 failed to reach a steady-state or periodic solution under the defined computer memory 18 usage and calculation time, and led to complex solutions, we gradually decreased 19 each conductance or the time constant from its original value to the one we calculated 20 under the defined memory usage and time. The number of solutions that resulted in 21 "Wake" between 0.001 and 1 times of its original value was counted for each 22 bifurcation parameter over the entire parameter sets.

23

1 Bifurcation analysis under KO condition

We first conducted parameter searches and subsequent bifurcation analysis under KO conditions by setting the target channel's conductance to zero. The rest of the channel conductance and/or the time constant were generated from the same exponential parameter space as the wild-type parameter search.

6

7 Animals and sleep phenotyping

8 All experimental procedures and housing conditions involving animals and their care 9 were approved by the Animal Care and the Use Committee of Graduate School of 10 Medicine, the University of Tokyo or by the Animal Care and Use Committee of the 11 RIKEN Kobe Institute, and all of the animals were cared for and treated humanely in 12 accordance with the Institutional Guidelines for Experiments using animals. All mice 13 were given food and water ad libitum, and kept at an ambient temperature and 14 humidity. The light was controlled under 12-hr light/12-hr dark cycle. Creation and sleep phenotyping of Cacna1g (set 2), Cacna1h (set 2), Atp2b3 (set 1 in UT), Atp2b3 15 16 (set 2), Camk2a, Camk2a (set 2), Camk2b (set 1), Camk2b (set 2), Camk2g, and 17 *Camk2d* were done in University of Tokyo by using C57BL/6N mice (n = 101) as 18 control information (Figures 3D-3F; Figures 7J-7M). All other experiments were 19 done in RIKEN by using the sleep phenotype of C57BL/6N mice (n = 108) published 20 in our previous paper as control information (Sunagawa et al., 2016).

21

1 Design of target sequences for guide RNA (gRNA)

2 The target sequences for Kcnma1, Kcnn1, Kcnn2, Kcnn3, Kcnn4, Kcnt1, Kcnt2, 3 Kcnu1 (Figures S2A-S2H), Cacna1a, Cacna1b, Cacna1c, Cacna1d, Cacna1e, 4 Cacna1f, Cacna1g (set 1 and 2), Cacna1h (set 1 and 2), Cacna1i, and Cacna1s 5 (Figures S4A-S4L) were designed using the on-line CRISPR guide RNA Design tool 6 (Ma et al., 2013) (http://cas9.cbi.pku.edu.cn/index.jsp) or the mm10 CRISPR/Cas9 7 database (Sunagawa et al., 2016) (http://www.crispr.riken.jp/). Possible off-target 8 sequences within the mouse genome for each target sequence were checked using 9 the CRISPR Design Tool (Ran et al., 2013) (http://tools.genome-engineering.org). 10 The alternative target sequences (set 2) for Kcnn2, and Kcnn3 (Figures S2J 11 and S2K), and the target sequences for Atp2b1, Atp2b2, Atp2b3 (set 1 and 2), 12 Atp2b4, Camk2a (set 1 and 2), Camk2b (set 1 and 2), Camk2g, and Camk2d 13 (Figures S7A-S7I, and S7O) were selected from the list resulting from the mm10 14 CRISPR/Cas9 database (Sunagawa et al., 2016) (http://www.crispr.riken.jp/).

15

16 gRNA synthesis

17 Oligonucleotides (listed below, Hokkaido System Science) containing the target 18 sequences for *Kcnma1* (Figure S2A) were annealed and inserted into the *Bbs*I sites 19 downstream of the U6 promoter on the pX330 plasmid (Cong et al., 2013; Ran et al., 20 2013) (Addgene, #42230). The resulting vectors were designated pX330-Kcnma1-1, 21 pX330-Kcnma1-2, and pX330-Kcnma1-3. The gRNA templates for Kcnma1 were 22 simultaneously fused to the T7 promoter and amplified from the pX330-Kcnma1-1, 23 pX330-Kcnma1-2, and pX330-Kcnma1-3 vectors by PCR using the primers listed 24 below (Hokkaido System Science) (Wang et al., 2013).

1 Alternatively, the gRNA templates for *Kcnn1*, *Kcnn2*, *Kcnn3*, *Kcnn4*, *Kcnt1*, 2 Kcnt2, Kcnu1 (Figures S2B-S2H, S2J, and S2K), Cacna1a, Cacna1b, Cacna1c, 3 Cacna1d, Cacna1e, Cacna1f, Cacna1g, Cacna1h, Cacna1i, Cacna1s (Figures 4 S4A-S4L), Atp2b1, Atp2b2, Atp2b3, Atp2b4, CaMK2a, Camk2b, Camk2g, and 5 Camk2d (Figures S4A-S4L, S7A-S7I and S7O) were directly synthesized and fused 6 to the T7 promoter by PCR. First, partial fragments of the gRNA templates including 7 each target sequence were amplified from the pX330 plasmids (Addgene, #42230) by 8 PCR with the Common Reverse primer (5'-AAAAGCACCGACTCGGTGCC-3', 9 Hokkaido System Science) (Wang et al., 2013) and Forward primer-1 for each target 10 sequence (Table S2, Hokkaido System Science). Subsequently, the T7 11 promoter-fused gRNA templates were amplified from the diluted PCR products by 12 PCR with the Common Reverse primer and Forward primer-2 for each target 13 sequence (Table S2, Hokkaido System Science). 14 The T7 promoter-fused gRNA PCR fragments were used as the template for in vitro transcription using the MEGAshortscript T7 kit (Life Technologies). The 15

16 gRNAs were purified using the MEGAclear kit (Life Technologies).

17

18 Oligonucleotide sequences for the Kcnma1 target sequences

- 19 Kcnma1-1:
- 20 Forward oligonucleotide: 5'-CACCGATGGATGCGCTCATCATAC-3'
- 21 Reverse oligonucleotide: 5'-AAACGTATGATGAGCGCATCCATC-3'

22 Kcnma1-2:

1	Forward oligonucleotide: 5'-CACCGATAAACAATGGCTCCAGCC-3'
2	Reverse oligonucleotide: 5'-AAACGGCTGGAGCCATTGTTTATC-3'
3	Kcnma1-3:
4	Forward oligonucleotide: 5'-CACCGTCTATGAAGTATATTACGA-3'
5	Reverse oligonucleotide: 5'-AAACTCGTAATATACTTCATAGAC-3'
6	
7	Oligonucleotide sequences for T7-gRNAs of Kcnma1
8	Common Reverse oligonucleotide for T7-gRNAs:
9	5'-AAAAGCACCGACTCGGTGCC-3' (Wang et al., 2013)
10	Kcnma1-1 Forward oligonucleotide:
11	5'- GGGCCTAATACGACTCACTATAGGATGGATGCGCTCATCATAC-3'
12	Kcnma1-2 Forward oligonucleotide:
13	5'- GGGCCTAATACGACTCACTATAGGATAAACAATGGCTCCAGCC-3'
14	Kcnma1-3 Forward oligonucleotide:
15	5'- GGGCCTAATACGACTCACTATAGGTCTATGAAGTATATTACGA-3'
16	
17	Cas9 mRNA synthesis
18	p3s-Cas9HC (Cho et al., 2013) (Addgene, #43945), which includes a T7
19	promoter-fused Cas9 coding region, was digested with XbaI (TaKaRa), and used as

1 the template for *in vitro* transcription using the mMESSAGE mMACHINE T7 kit (Life

2 Technologies). The Cas9 mRNA was purified using the MEGAclear kit (Life

3 Technologies).

4

5 One-cell embryo microinjection

6 C57BL/6N females (4-6 weeks old, CLEA Japan Inc) were superovulated and mated 7 with C57BL/6N males (CLEA Japan Inc). Fertilized eggs were collected from the 8 ampulla of the oviduct of plugged C57BL/6N females by micro-dissection, and kept in 9 KSOM medium (Merck Millipore or ARK Resource) in a 5% CO₂ incubator at 37°C. Cas9 mRNA (100 ng/µl) and gRNAs (150 ng/µl in total) were co-injected into the 10 11 cytoplasm of fertilized eggs in M2 medium (Merck Millipore or ARK Resource) at 12 room temperature. Details of the cytoplasmic injection were reported previously 13 (Sumiyama et al., 2010). After microinjection, the embryos were cultured for 1 hr in 14 KSOM medium (Merck Millipore or ARK Resource) in a 5% CO₂ incubator at 37°C, 15 and 15-30 embryos were then transferred to the oviducts of pseudopregnant female 16 ICR mice.

17

18 Genotyping of knockout mice by quantitative PCR (qPCR) and sequencing

The genomic DNA of wild type and KO mice was prepared from their tail using the DNeasy Blood & Tissue Kit (QIAGEN), according to the manufacturer's instructions. qPCR for genotyping was performed using the ABI PRISM 7900/QuantStudio 7 Flex (Applied Biosystems) and the SYBR Premix Ex Taq GC (TaKaRa). Primers for qPCR (Table S3, Hokkaido System Science) were annealed to the targeting sequences. The absolute target site abundance was calculated using a standard curve obtained from wild-type genomic DNA. The amount of *Tbp* (Tsujino et al., 2013) was
quantified and used as an internal control. When we could not confirm KO genotype
by qPCR, we performed sequencing or 2nd qPCR using the alternative primer which
was independent of 1st qPCR. We performed sequencing to the mice in which we
could not confirm KO genotype by the 2nd qPCR.

Genotyping by sequencing was performed for mice which the relative amount
of intact DNA in qPCR was higher than 0.2 in all of three target sites. The 0.7 ~ 1.8 kb
around each target sequence was amplified by PCR with Ex Taq (TaKaRa) and the
primers (Table S4, Hokkaido System Science). The fragment was subcloned into
pGEM-T-easy vector (Promega), and sequenced.

11

12 Pharmacological administration with SSS recording

13 C57BL/6J or C57BL/6N mice received an i.p. injection of PCP hydrochloride 14 synthesized according to the previous report (Prashad et al., 2005) (dissolved with 15 normal saline to be 13.3 and 26.7 mg/kg at 200 µl for Figures 4A and 4B; Figures 16 S5A and S5B) or MK-801 maleate (Tocris Bioscience, Catlog# 0924, Lot# 9A/151915, 17 diluted with natural saline to be 0.2, 2 and 20 mg/kg at 200 µl for Figures 4C and 4D; 18 Figures S5C and S5D) at ZT2, respectively. For chronic treatment of MK-801, the 19 drug (Wako, 136-17383, Lot# WEL3580) was diluted to sterile water to be 16, 32, 64 20 and 96 mg/l and treated to C57BL/6J mice as drinking water (Figures S5E-S5G). For 21 the CUBIC analysis, 64 mg/l of MK-801 was given to the treated group as drinking 22 water (Figure 5; Figures 6A-6D; Figures S6A-S6E).

23

1 Pharmacological administration with EEG/EMG recording

C57BL/6N mice implanted with telemetry EEG/EMG recording device received an i.p.
injection of MK-801 maleate (Tocris Bioscience, Catlog# 0924, Lot# 1A/15826,
diluted with natural saline to be 2 mg/kg at 200 µl for Figures 4E and 4F) or natural
saline at ZT2, respectively. The recorded EEG/EMG data were analysed by the
FASTER method. Further information on implantation of telemetry devices and data
analysis of EEG/EMG recordings has been published previously (Sunagawa et al.,
2013).

9

10 Sleep deprivation

C57BL/6N mice and KO mice implanted with telemetry EEG/EMG recording device
were sleep deprived by gentle handling for eight hours. The deprivation started on
ZT4. Subsequently, mice were left to sleep freely for 2 days. The recorded EEG/EMG
data were analysed by FASTER.

15

16 Brain sampling from Arc-dVenus mice with MK-801 administration

The Arc-dVenus transgenic mice (line D) (Eguchi and Yamaguchi, 2009) were housed in the SSS chamber under 12-hr light/12-hr dark cycle (LD conditions). On day 0, MK-801 (Wako Pure Chemical Industries Ltd., 136-17383, Lot# WEL3580) diluted to sterile water to be 64 mg/l was given to the treated group as drinking water and sterile water to the control group. On day 5, the light condition was changed to constant darkness, and the animals with or without MK-801 administration were sacrificed at circadian time (CT) 4, 10, 16 and 22. We prepared samples from multiple

1 animals within ~30 min. The mice were deeply anesthetized with an overdose of 2 pentobarbital (>100 mg/kg, i.p.; Somnopentyl, Kyoritsu Seiyaku), then transcardially 3 perfused with PBS containing ~10 U/ml heparin (Wako Pure Chemical Industries Ltd.) 4 to flush the blood vessels, followed by perfusion with 4% (w/v) paraformaldehyde 5 (PFA) in PBS (pH 7.4) for fixation. The dissected brains were then post-fixed with 4% 6 PFA at 4°C for 24 hr, washed with PBS, and immersed in 20% (w/v) sucrose/PBS for 7 at least one day. After the sucrose/PBS replacement, the samples were stored in 8 O.C.T. compound at -80°C until use.

9

10 **CUBIC protocol**

11 The two CUBIC reagents (ScaleCUBIC-1 and -2) were prepared as previously 12 reported (Susaki et al., 2014; Susaki et al., 2015). The whole-brain clearing protocol 13 was slightly modified from the previous report. Each fixed brain was immersed in 1:1 14 diluted reagent-1:water with 2.5 µM nucleic acid stain TOPRO3 (Life Technologies, 15 T3605) at 37°C with gentle shaking for 3 hr, after which the solution was removed and 16 the sample immersed in 7 ml of fresh reagent-1 with 2.5 µM TOPRO3. The reagent 17 was replaced by fresh reagent-1 with 2.5 µM TOPRO3 on days 1 and 3. On day 6, the 18 treated brain was washed with PBS several times at room temperature with gentle 19 shaking overnight, and then immersed in 4 ml of 1.5 M NaCl/PBS with 2.5 µM 20 TOPRO3 for 2 days for additional staining. After staining, the sample was washed 21 with PBS, immersed in 20% sucrose/PBS for at least 2 hr at 4°C, and stored in O.C.T. 22 compound at -80°C until use. Two days before imaging, the sample was thawed, 23 washed with PBS, immersed in 4 ml of 1:1 diluted reagent-2:PBS, and immersed in 4 24 ml of reagent-2 for 2 days. After imaging, the sample was again washed with PBS, 25 immersed in 20% sucrose/PBS, and stored in O.C.T. compound at -80°C.

1

2 Microscopy

3 Whole-brain fluorescence images were acquired with an LSFM (Ultramicroscope, 4 LaVision BioTec) as reported previously (Dodt et al., 2007; Susaki et al., 2014; 5 Susaki et al., 2015). Venus signals were imaged using a 488 nm laser light 6 (Coherent Sapphire488LP-100) and emission filters 525/50 (Semrock). TOPRO3 7 signals were imaged using 588 nm laser light (Coherent Sapphire588LP-50) and 8 emission filters 690/50 (Chroma), with a sCMOS camera (Andor Neo 5.5) and a 9 macrozoom microscope (Olympus MVX-ZB10) with a 0.63 × objective lens (Olympus 10 MVPLAPO0.63X). Each plane was illuminated from both the right and left sides of the 11 sample, and the merged image was saved. Three-dimensional images were collected 12 as a Z stack series: 10-µm steps x 668-847 planes for the dorsal-ventral direction and 13 10-µm steps x 629-858 planes for the ventral-dorsal direction, with 800-ms 14 illuminations for Venus and 50-ms illuminations for TOPRO3.

15 Images of in situ hybridization samples were acquired as 8-bit TIFF images with 16 FV1200/IX83 inverted confocal microscopy (Olympus) with a 10 × (Olympus 17 UPLSAPO 10×/0.4) objective lens, 405/473/559 nm lasers and PMT detectors. 4.68 18 µm × 6 plane images were collected and stacked. We also used an upright 19 fluorescent microscopy (BX51, Olympus) with a 4 × (Olympus UPIanApo 4×/0.16) 20 and 10 × (Olympus UPIanFL N 10×/0.3) objective lens, a CCD (Olympus DP30BW) or 21 a cMOS (Andor Zyla) camera, and appropriate excitation/emission filter sets for DAPI, 22 Alexa-Fluor 488 and Cy3. A series of images covering all areas of a section were 23 acquired and stitched with pairwise stitching plugin of ImageJ (Preibisch et al., 24 2009).

1 Image analysis

For each brain sample of CUBIC analysis, 3D NIfTI-1 files were generated from TIFF
stacks acquired in opposite directions, merged and registered as described
previously (Susaki et al., 2014).

5 To normalize the Arc-dVenus signals, we extracted the pixels located only in 6 brain regions, by aligning the NIfTI-1 images with the Allen Brain Atlas (Lein et al., 7 2007). We then calculated the median intensity of these pixels for each brain, and 8 normalized the data so that all of the brain samples had the same median intensity.

9 Overall brain images (Figure S6A) were obtained by applying the
10 normalization factors to the raw TIFF stacks and reconstructing them using the Imaris
11 software (Bitplane).

MK-801(+) and MK-801(-) were compared using the registered and
normalized NIfTI-1 files. For each time point, we first calculated the average
Arc-dVenus signal for both groups, using the "fsImerge –t" and "fsImaths –Tmean"
tools in the FSL software (Jenkinson et al., 2012). We then subtracted the MK-801(-)
average brain from the MK-801(+) brain at each time point using "fsImaths –sub."

17 The hyper-activated cells were analyzed using the Fiji software (Schindelin 18 et al., 2012). For each horizontal slice of a given brain sample, we converted the 19 image to 8-bit and successively ran the "Find Edges," "setThreshold" (with 20 parameters 128, 255), "Threshold," and "Analyze Particles" (with size = 10-500, 21 circularity = 0.3-1.0) commands. The number of hyper-activated cells in one brain 22 sample was the sum of the number of cells identified in each of its slices (Figure 23 **S6C**). When processing each slice, we also saved an image containing only the 24 detected hyper-activated cells, so that we can reconstruct 3D images of cell

hyper-activation throughout the brain. This detection method is shown for two
 MK-801(-) and MK-801(+) slices in Figure 5A, right.

3 We then analyzed region-specific pattern in cell hyper-activation. Querving 4 specific regions relies on images aligned to the Allen Brain Atlas. These images do 5 not have single-cell resolution, and the Fiji-based method cannot be applied. Instead, 6 we created a binary mask for each region, and applied it to the reconstructed 3D 7 images showing only hyper-activated cells. The intensity within each region can be 8 used to estimate the number of hyper-activated cells in this region. For the whole 9 brain, the relation between the total intensity *i* and the number of cells *n* is given by *n* = $0.0123 \times i$, $R^2 = 0.94688$ (Figure S6D). For each region, using the average intensity 10 i_{ava} and the voxel dimension (25.8×25.8×40µm), we can therefore calculate n_{val} , the 11 number of cells per mm³ as, n_{vol} = 461.961 × i_{ava} (Figure 5A and 5C-5E, right 12 13 panels; Figure 6D; Figure S6E, right panel).

14 Brain regions were then clustered based on their normalized intensity across 15 the four time-points with or without MK-801 administration (Figure 5B), using the 16 heatmap.2 function in the gplots R package (Warnes et al., 2014), with Euclidean 17 distance and the "ward.D2" clustering method. For each cluster, a binary mask was 18 created, and the cluster-specific hyper-activated cell signal was extracted (Figures 19 5C-5E, left panels; Figure S6E, left panels). Using the formula above, we also 20 estimated the number of hyper-activated cells in each cluster (Figures 5C-5E, right 21 panels; Figure S6E, right panels). Using Cytoscape (Shannon et al., 2003), we 22 also constructed a tree showing the hierarchical annotation structure of the Allen 23 Brain Atlas. Nodes represent brain regions, and are colored based on the cluster they 24 belong to Figure 5F. Regions with low signal (i.e. at most two non-zero values over 25 the twenty brain samples) were excluded from the clustering and are colored in light 26 gray. Spatio-temporal profiling data of the estimated number of hyper-activated cells
in each brain region are available in **Table S6**. To avoid showing the cell in multiple
 clusters due to the tree structure of the brain annotation, only leaf regions are used in
 the image reconstruction when we construct 3D-reconstituted brain images (Figures
 5C-5E).

5 Cell hyper-activation is not only region-specific, but also layer-specific. To 6 investigate this, we also created binary masks for layers in the cerebrum. The same 7 analysis as above was performed for layers 1, 2-3 and 4-6. We were therefore able to 8 extract the hyper-activated cell signal for these three layers, as well as estimate the 9 number of hyper-activated cells (**Figures 6A-6C**).

10 For cell counting analysis of FISH images, Layer 4, 5 and 6 of cerebral cortex 11 in a section image (from visual to motor areas, see **Figure S6G**) were manually 12 cropped. The background signals of *c-Fos* channel were eliminated by setting an 13 appropriate threshold. To detect sustainably activated neurons of which soma was 14 strongly stained by the *c*-Fos probes, the particle analysis of ImageJ with a range of 15 soma's size was performed. The resulted mask image was manually curated to 16 eliminate noise signals and add undetected active cells showing obvious 17 over-threshold signals, and also used to detect double-positive cells by merging Nr2b, 18 Vglut1/2, or Gad1/2 channels from corresponding sections. To count Nr2b, Vglut1/2, 19 Gad1/2 or DAPI-stained cells, the background signals of each channel were 20 eliminated in the similar way, and then the images were subjected to the particle 21 analysis of ImageJ after changing to a binary image and calculating watershed.

To show the significant difference of the numbers of *c-Fos* and *Nr2b* co-expression cells between MK-801 (+) and MK-801 (-), these cell counts were used for *F*-test. To confirm that co-expression of *c-Fos* and *Nr2b* were not a coincidence, we performed Chi-squared tests against these cell counts. All statistical analyses

were performed via R version 3.1.0. *p* < 0.05 were considered significant in *F*-tests
 and Chi-squared tests.

3

4 Fluorescence in situ hybridization with MK-801 administration

Male C57BL/6N mice (6-week-old) purchased from CLEA Japan, INC were used.
MK-801 maleate (Tocris Bioscience, Lot# 9A/151915) was dissolved in normal saline
and diluted to be 2 mg/kg at 200 µl. At ZT2, 200 µl of this solution or normal saline
were injected intraperitoneally into each mouse. The mice were sacrificed at ZT5, and
their brain was used for *in situ* hybridization as follows.

10 The fluorescence in situ hybridization (FISH) protocol was based on 11 TAI-FISH (Xiu et al., 2014) with some modifications. RNase-free reagents and 12 materials were used during the procedure. For sample preparation, each mouse was 13 anesthetized with an overdose of pentobarbital (>100 mg/kg, i.p.; Somnopentyl, 14 Kyoritsu Seiyaku), then transcardially perfused with cold PBS to flush the blood 15 vessels, followed by perfusion with 4% (w/v) paraformaldehyde (PFA) in PBS for 16 fixation. The dissected brain was then post-fixed with 4% PFA at 4°C overnight, 17 washed with PBS, and immersed in 20% sucrose/PBS for one day.

18 Probes against Nr2b were amplified from mouse brain cDNA (9-week-old 19 C57BL6/N male brain cDNA, synthesized by ourselves). Probes against *c-Fos*, 20 Vglut1, Vglut2, Gad1, and Gad2 (kindly provided by Dr. Kazunari Miyamichi) were 21 amplified from mouse brain cDNA (first strand cDNA mouse brain MD01, GenoStaff). 22 All the probe fragments were cloned into pCR-BluntII-TOPO vector (Life 23 Technologies) for storage, and *in vitro* transcription (IVT) templates were generated 24 by PCR using the cloned vector. All primers used for cloning and IVT reactions are 25 listed in **Table S5**. The RNA probes labelled with digoxigenin (DIG) -UTP or

1 fluorescein (Flu) -UTP (Roche) were generated by IVT with T3 polymerase (Roche), 2 purified on an Illustra ProbeQuant G-50 column (GE Healthcare). The resulted 3 concentrations of the IVT products were about 50~100 ng/µl, and approximately the 4 same amount of probes for the same gene were mixed for usage. Probe mixes used 5 in this study are *c*-Fos-1 and 2 (*c*-Fos probes), Nr2b-1, 2 and 3 (Nr2b probes), 6 Vglut1-1, Vglut2-1, 2, and 3 (Vglut1/2 probes), or Gad1-1, 2, Gad2-1, 2, and 3 7 (Gad1/2 probe). Pairs of probe mixes used in this study were c-Fos-Flu and Nr2b-DIG, 8 c-Fos-DIG and Vglut1/2-Flu, or c-Fos-DIG and Gad1/2-Flu, respectively.

9 For fluorescence *in situ* hybridization, sections (30-40 µm thickness) were 10 prepared with a cryostat under RNase-free conditions. Sagittal sections from 3.8-3.9 11 mm inside from lateral end of the right hemisphere were used for the analysis in this 12 study. Each section was collected into PBS for washing O.C.T. compound, promptly 13 put on a MAS-coated slide glass (Matsunami glass ind., LTD., Japan) and dried up. 14 Then the sections were washed with PBS and treated with 10 µg/ml of protease K in 15 10 mM Tris-Cl (pH 7.4) and 1 mM EDTA for 10 min at 37°C. The treated sections 16 were post-fixed with 4% PFA in PBS for 10 min at room temperature and washed with 17 PBS. The sections were then acetylated with 0.25% acetic anhydride (v/v) in TEA 18 solution (1.3% [v/v] triethanolamine, 0.25% [v/v] HCI) for 15 min and washed with 19 PBS. The samples were dried up again and subjected to hybridization by incubation 20 in the hybridization solution (50% formamide [v/v], 0.6 M NaCl, 10 mM Tris-Cl [pH 8.0], 21 5 mM EDTA, 0.3 mg/ml yeast tRNA, 0.1 mg/ml heparin, 1× Denhardt's solution, 0.1% 22 Tween 20 [v/v] and 0.25% SDS [w/v]) with a pair of probe mixes (1:100-1:200 dilution) 23 overnight at 60°C. After hybridization, the sections were rinsed with 5× SSC, and then 24 washed once in 50% (v/v) formamide in 2× SSC, once in 2× SSC, and twice in 0.2× 25 SSC for each 20 min at 65°C.

1 Two-color signal detection with the Tyramide Signal Amplification system was 2 performed at room temperature except for the antibody reactions (indicated below). 3 The hybridized sections were incubated in blocking buffer (1% [w/v] blocking reagent 4 [Roche 11096176001] in 100 mM maleic acid, 150 mM NaCl and 0.3% Tween 20, 5 adjusted to pH 8.0 with NaOH) for 1 hr, and then in the buffer containing anti-Flu-HRP 6 (PerkinElmer NEF710001EA, 1:250) overnight at 4°C. On the second day, the 7 sections were washed three times in PBS containing 0.3% Triton X-100 (PBST) for 10 8 min each, then incubated in the amplification solution with biotin tyramide (1:100, 9 PerkinElmer, NEL749A001KT) for 20 min, and washed once with PBST for 10 min. 10 HRP was inactivated with 2% (w/v) sodium azide in PBS for 20 min, followed by three 11 washes in PBST for 10 min each to completely remove the sodium azide. The 12 sections were then incubated in the blocking buffer with anti-DIG-HRP (Roche 13 11207733910, 1:500) and streptavidin-Alexa Fluor 488 (Life Technologies, 1:250) 14 overnight at 4°C. On the third day, the sections were washed three times in PBST 15 and incubated in the amplification solution with cyanine 3-labeled tyramide (1:100, PerkinElmer NEL744001KT) for 20 min, and further washed twice with PBST for 10 16 17 min. The sections were embedded with an agent (Vectashield mounting medium with 18 DAPI, Vector Laboratories Inc.). We collected the FISH sections of four saline-treated 19 and four MK-801-administrated mice from two independent experiments and two of 20 them with significant FISH signals were selected for further cell counting experiments.

21

22 Statistical analyses

23 Statistical analyses were performed by Microsoft Excel or R version 3.1.0.

For sleep phenotype analyses of CRISPR KO mice, normality was evaluated
by Kolmogorov-Smirnov test at the significance level of 0.05. The homogeneity of

variance for each group was evaluated by Bartlett's test when all the member of the
group was normal, or Levene's test when not. In both tests, significance level was set
to 0.05. The sleep phenotype of every KO strain was compared to the control
(C57BL/6N, male, n=108 or n=101 in UT) by Dunnett's test when the group has
homogenous variance and Dunnett's Modified Tukey-Kramer pairwise multiple
comparison test when not.

7 To estimate the sample size for phenotype evaluation, we compared the 8 sleep duration control dataset Xc (C57BL/6N, male, n=108 or n=101 in UT) to a 9 simulated dataset Xs which have the same distribution except a mean of 1 SD (50.8 min) less or much than control ($\widetilde{Xs} = \widetilde{Xc} \pm 50.8$). We randomly sampled k ($2 \le k \le 20$) 10 11 data from Xs and compared with Xc by Dunnett's test with the significance level of 12 0.05. This test was repeated 1000 times for one k. We calculated the rate of p < 0.0513 as sensitivity for each k from 2 to 20. According to this calculation, we found that the 14 sample size $k \ge 2$ provides more than 25% sensitivity whereas the sample size $k \ge 6$ 15 provides more than 50% sensitivity. In this study, we selected the sample size $k \ge 2$ to 16 achieve more than 25% sensitivity for the CRISPR knockout study.

For the analysis of the experiments by the EEG/EMG recording, means of two sets of data were evaluated. First, the normality was tested by Shapiro test at the significant level of 0.05. When the normality is confirmed in both groups, the homogeneity of variance was tested by *F*-test at the significance level of 0.05. When two groups were normal distributions with equal variance, Student's *t*-test, when the groups are normal distributions without equal variance, Welch's *t*-test, otherwise two-sample Wilcoxon test was applied.

The i.p. injection was performed blindly without informing the injector the content of the drug. Randomization was not performed for processing data.

- 1 In this study, p < 0.05 was considered as significant (*p < 0.05, **p < 0.01,
- ***p < 0.001 and n.s. for not significant evaluations).

1 Supplemental Figure Legends

- Figure S1. The averaged-neuron model recapitulates slow-wave-sleep and
 awake firing patterns, related to Figure 1.
- (A) The ionic currents through NMDA receptors, voltage-gated Ca²⁺ channels,
 Ca²⁺-dependent K⁺ channels, GABA_A receptors, and Ca²⁺ efflux show alternating
 bursting and silent cycles in representative slow-wave-sleep firing pattern shown in
 Figure 1B.
- 8 (**B-G**) Representative awake firing patterns generated by lowering the parameter
- 9 value of g_{KCa} (**B**), g_{NMDA} (**C**), g_{Ca} (**D**), τ_{Ca} (**E**), $g_{\text{NMDA}} / g_{\text{Ca}} / \tau_{\text{Ca}}$ (**F**), or $g_{\text{KCa}} / g_{\text{NMDA}} / g_{\text{Ca}} / g_{\text{Ca$
- 10 $\tau_{Ca}(G)$, respectively. The membrane potential and ionic currents through NMDA
- 11 receptors, voltage-gated Ca²⁺ channels, Ca²⁺-dependent K⁺ channels, and GABA_A
- 12 receptors show bursting behaviors whereas intracellular Ca²⁺ concentration and Ca²⁺
- 13 efflux exhibit more stable behaviors.
- 14 (H-J) The parameter search under NMDA receptor KO (H), voltage-gated Ca²⁺
- 15 channel KO (I) and GABA_A receptor KO (J) conditions yielded 1,176, 1,011 and 1,069
- 16 parameter sets that exhibited slow-wave-sleep firing patterns. The normalized
- 17 membrane potential and intracellular Ca^{2+} are shown for all parameter sets. Six
- 18 cycles of bursting and silent phases for each slow-wave-sleep firing pattern are drawn.
- 19 The membrane potential and intracellular Ca^{2+} are normalized to have a mean of zero
- 20 and one SD for each parameter set.
- 21 (K) Summary of bifurcation analysis of parameter sets that elicited the
- 22 slow-wave-sleep firing pattern under GABA_A receptor KO conditions. Graph shows
- 23 the percentage of the slow-wave-sleep parameter sets that switched from
- slow-wave-sleep to awake firing patterns when the indicated parameter value was

- 1 decreased to 10⁻³ times its original value. Red text indicates the conductance and
- 2 time constant related to the Ca^{2+} hyperpolarization pathway.
- 3 (L) Discovery rate of slow-wave-sleep firing patterns during parameter searches
- 4 under wild-type or ion channel KO conditions.
- 5

Figure S2. Impairment of Ca²⁺-dependent K⁺ channels decreases sleep duration, related to Figure 2.

(A-H) Target sequences of the gRNAs for KO mice of the Ca²⁺-dependent K⁺ channel 3 4 family [Kcnma1 (A), Kcnn1 (B), Kcnn2 (set 1, C), Kcnn3 (set 1, D), Kcnn4 (E), Kcnt1 5 (F), Kcnt2 (G), and Kcnu1 (H)]; each gene had three target sequences. Mouse 6 genomic sequence data were obtained from GRCm38/mm10 via the UCSC Genome 7 Browser (Rhead et al., 2010) (http://genome.ucsc.edu/). Colored letters (blue, orange, 8 and green) show the 20-base target sequences. The target sequences were 9 designed on the sense (+) or antisense (-) strand of genomic DNA. (I) Distributions of sleep duration of the KO mice of Ca^{2+} -dependent K⁺ channels 10 11 compared in a different time of the day. Morning and night are defined as ZT0 to ZT12 12 and ZT12 to ZT24, respectively. Histogram at the top of each chart shows the results 13 from C57BL/6N male mice (n = 108). The error bar is the SEM for each strain, and the

14 black dashed vertical line and gray shaded area are the mean and 1 SD range from

15 the recordings of C57BL/6N male mice. *p < 0.05, **p < 0.01, ***p < 0.001, compared

- 16 to C57BL/6N male mice, Dunnett's test.
- 17 (J and K) Target sequences of the gRNAs for independent CRISPR/Cas9 probe sets
- 18 for knocking out genes of *Kcnn2* (set 2, **J**) and *Kcnn3* (set 2, **K**); each gene had three
- 19 target sequences. Mouse genomic sequence data were obtained from
- 20 GRCm38/mm10 via the UCSC Genome Browser (Rhead et al., 2010)
- 21 (http://genome.ucsc.edu/). Colored letters (blue, orange, and green) show the
- 22 20-base target sequences. The target sequences were designed on the sense (+) or
- 23 antisense (-) strand of genomic DNA.

- 1 (L) Sleep-duration phenotype based on EEG/EMG for wild-type mice (n = 4) and
- 2 $K_{Ca}2.2$ (Kcnn2) KO mice (n = 9) generated by triple-target CRISPR. Average P_{ws} (left)
- 3 and P_{sw} (right) are shown in bar graphs. Error bars: SEM.

Figure S3. Genotyping of KO mice by CRISPR/Cas9 injection, related to Figure 2 2.

The genotype of KO mice for the Ca^{2+} -dependent K⁺ channel family [*Kcnma1* (**A**), 3 4 Kcnn1 (B and C), Kcnn2 (D, K, M and N), Kcnn3 (E and L), Kcnn4 (F), Kcnt1 (G), 5 *Kcnt2* (**H** and **I**) and *Kcnu1* (**J**)]; The relative amount of intact DNA for each target sequence was measured by quantitative PCR (qPCR). The genomic DNA was 6 7 purified from the tail of each mouse. The relative amount of intact DNA for each target 8 sequence was scaled so that the level in a wild-type mouse was defined as 100%. 9 Genotyping by sequencing was done for a *Kcnn1* KO mouse (**C**), three *Kcnt2* KO 10 mice (I), and seven *Kcnn2* KO mice (N). The sequences of mutant alleles in each 11 mouse are shown. The target sites are colored and underlined. The mutations are 12 labeled in red. The numbers of each detected allele are put in parentheses. The KO mice, which are confirmed by 1st gPCR, 2nd gPCR or sequencing, are labeled in red, 13 14 green or blue, respectively.

15

Figure S4. Impairment of voltage-gated Ca²⁺ channels decreases sleep duration, related to Figure 3.

(A-L) Target sequences of the gRNAs for KO mice of the voltage-gated Ca²⁺ channel 3 4 family [Cacna1a (A), Cacna1b (B), Cacna1c (C), Cacna1d (D), Cacna1e (E), Cacna1f 5 (F), Cacna1g (set 1, G), Cacna1h (set 1, H), Cacna1i (I), Cacna1s (J), Cacna1g (set 2, 6 **K**) and *Cacna1h* (set 2, L)]; each gene had three target sequences. Mouse genomic 7 sequence data were obtained from GRCm38/mm10 via the UCSC Genome Browser 8 (Rhead et al., 2010) (http://genome.ucsc.edu/). Colored letters (blue, orange, and 9 green) show the 20-base target sequences. The target sequences were designed on 10 the sense (+) or the antisense (-) strand of genomic DNA. (D) The lowest variant 11 (magenta) is an additional variant of Cacna1d predicted on C57BL/6 genome 12 sequence (GenBank, http://www.ncbi.nlm.nih.gov/nuccore/). Our triple gRNAs are 13 designed to target all variants. A green square shows the region which used the 14 targeted disruptions of Cacna1d gene in previous reports (Namkung et al., 2001; 15 Platzer et al., 2000). There is a possibility that two variants (magenta) express in the 16 KO mice in these reports.

(M-T) The genotype of KO mice for the voltage-gated Ca²⁺ channel family [*Cacna1b*(M), *Cacna1e* (N), *Cacna1f* (O), *Cacna1g* (set 1, P), *Cacna1h* (set 1, Q), *Cacna1i* (R), *Cacna1g* (set 2, S) and *Cacna1h* (set 2, T)]; the relative amount of intact DNA for
each target sequence was measured by quantitative PCR (qPCR). The genomic DNA
was purified from the tail of each mouse. The relative amount of intact DNA for each
target sequence was scaled so that the level in a wild-type mouse was defined as
100%.

(U) Genotyping by sequencing was done for three of *Cacna1h* KO mice (set 2). The
 sequences of mutant alleles in each mouse are shown. The target sites are colored

- 1 and underlined. The mutations are labeled in red. The numbers of each detected
- 2 allele are put in parentheses.

Figure S5. Impairment of NMDA receptors decreases sleep duration, related to Figure 4

(A) Dose-dependent sleep phenotype resulting from a single i.p. injection of
phencyclidine (PCP) to C57BL/6J mice at ZT2. Heatmaps show the sleep duration
(per hour) for all mice in three groups (0, 13.3, and 26.7 mg/kg). The 0 mg/kg group
was used as a control. Red bar at the top of the plot shows the period used for
comparing the parameters in Figure S5B.

8 (B) PCP significantly reduced the sleep duration and P_{ws} in a dose-dependent

9 manner for 5 hr (ZT2 to 6) after its i.p. injection. (Left) Average sleep duration. (Middle

10 and right) Average P_{ws} and P_{sw} . Error bars: SEM.

11 (C) Dose-dependent sleep phenotype of a single i.p. injection of MK-801 to C57BL/6J

12 mice at ZT2. Heatmaps show the sleep duration (per hour) for all mice in four groups

13 (0, 0.2, 2 and 20 mg/kg). The 0 mg/kg group was used as a control. Red bar at the top

14 of the plot shows the period used for comparing the parameters in **Figure S5D.**

15 (**D**) MK-801 significantly reduced the sleep duration and P_{ws} in a dose-dependent

16 manner for 5 hr (ZT2 to 6) after its i.p. injection. (Left) Average sleep duration. (Middle

17 and right) Average P_{ws} and P_{sw} . Error bars: SEM.

18 (E) Sleep phenotype during chronic administration of MK-801 to C57BL/6J mice.

19 Heatmaps show the sleep duration (per hour) for all mice in five groups, which were

20 given MK-801 in the drinking water at different concentrations (0, 16, 32, 64 and 96

21 mg/l). The 0 mg/l group was used as a control.

(F) Sleep duration (per hour) over 24 hr averaged over six days under the chronic
administration of MK-801. Red lines show the mean sleep duration at each time of

- 1 day for each group. Gray line shows the data for C57BL/6N male mice (n = 108). The
- 2 shaded area around the line is the SEM for each time point.
- 3 (G) Chronic administration of MK-801 decreased the sleep duration in a
- 4 dose-dependent manner. Panels show the averaged daily sleep duration, amplitude,
- 5 P_{ws} , and P_{sw} . Error bars: SEM.
- 6 *p < 0.05, **p < 0.01, ***p < 0.001, compared to control group in each experiment,
- 7 Dunnett's test or Dunnett's modified Tukey-Kramer pairwise multiple comparison test
- 8 (**B**, *P*_{ws}).
- 9

Figure S6. Impairment of the Ca²⁺-dependent hyperpolarization pathway increases neural excitation, related to Figure 5 and 6.

3 (A) Temporal variation of 3D-reconstituted brain images of TOPRO3-stained 4 Arc-dVenus Tg mice with and without the chronic administration of MK-801. After 5 measuring the sleep/wake phenotype under LD condition for 4 days, Arc-dVenus Tg 6 mice with (n = 3 per time point, the lower row) and without (n = 2 per time point, the point)7 upper row) the chronic administration of MK-801 were kept in the dark, and the brains 8 were sampled every 6 hr. The sampled brains were then cleared using the CUBIC 9 reagents. Images were acquired with light-sheet fluorescence microscopy (LSFM) 10 from both the dorsal-to-ventral (D-V) and ventral-to-dorsal (V-D) directions. 11 Representative images of each time point acquired from the D-V direction are shown. 12 Green and gray indicate Arc-dVenus and TOPRO3 signals, respectively. Data were 13 normalized so that the median intensity of the Arc-dVenus signals inside the brain 14 matched across all samples. CT: Circadian time. Bar: 2000 µm.

(B) Sleep duration (per hour) over 24 hr averaged over 4 days before the animals
were set in constant darkness for brain sampling. The lines show the mean sleep
duration at each time of day for each group. Gray line shows the data for C57BL/6N
male mice (n = 108). The shaded area around the line is the SEM for each time point.
The size of the dot denotes the *p*-values when the sleep duration is tested by
Dunnett's test or Dunnett's Modified Tukey-Kramer pairwise multiple comparison test
with the wild-type animal recording as a control.

(C) Quantification of hyper-activated cells. Hyper-activated cells were automatically
 extracted using a macro developed with the Fiji software. Left: Representative slice
 images at CT4 with and without the chronic administration of MK-801 are shown.

Middle: Magnified images are shown. Right: Blue and red indicate hyper-activated
 cells with and without the chronic administration of MK-801.

3 (D) Relationship between the intensity of the signals extracted from detected
4 hyper-activated cells and the number of detected hyper-activated cells in the whole
5 brain.

6 (E) Temporal variation of the number of hyper-activated cells in the purple cluster. 7 Left: 3D-reconstituted brain images of the Arc-dVenus signals extracted from cells 8 detected as hyper-activated and located in regions belonging to the purple cluster of 9 the heatmap (shown in purple). Gray indicates TOPRO3 signals. To avoid showing 10 the cell in multiple clusters due to the tree structure of the brain annotation, only leaf 11 regions are used in the image reconstruction. CT: Circadian time. Bar: 2000 µm. 12 Right: Estimated number of hyper-activated cells in the purple cluster and at each 13 time-point with (n = 3, red) or without (n = 2, blue) the chronic administration of 14 MK-801. Error bars: SEM.

(F and G) Images of FISH analysis (using *c-Fos* and *Nr2b* probes) of a sagittal
section from animals subjected to intraperitoneal saline (F) or MK-801 (G)
administration. The cropped regions shown in Figures 6E and 6F are indicated with
white boxes. Cortical areas between dashed lines were used for cell counting
analysis in Figure 6G. The corresponding cortical areas were also used for cell
counting analysis in Figure 6J. Bar: 1 mm

(H and I) Images of FISH analysis of the cerebral cortex from an MK-801-treated
mouse using *c-Fos* and either *Vglut1/2* (H) or *Gad1/2* (I) probes, respectively. The
cropped regions shown in Figures 6H and 6I are indicated with white boxes. Bar: 100
µm.

Figure S7. Impairment of Ca²⁺ pump decreases sleep duration and impairment 1 2 of CaMKII decreases sleep duration, related to Figure 7.

(A-H) Target sequences of the gRNAs for KO mice of the plasma membrane Ca²⁺ 3 4 ATPase and the calcium/calmodulin-dependent protein kinase family [Atp2b1 (A)]. 5 Atp2b2 (B), Atp2b3 (set 1, C), Atp2b4 (set 1, D), Camk2a (E), Camk2b (F), Camk2d 6 (G) and Camk2g (H)]; each gene had three target sequences. Mouse genomic 7 sequence data were obtained from GRCm38/mm10 via the UCSC Genome Browser 8 (Rhead et al., 2010) (http://genome.ucsc.edu/). Colored letters (blue, orange, and 9 green) show the 20-base target sequences. The target sequences were designed on 10 the sense (+) or antisense (-) strand of genomic DNA.

11 (I and O) Target sequences of the gRNAs for independent CRISPR/Cas9 probe sets 12 for KO mice of Atp2b3 (set 2, I), Camk2a and Camk2b (set 2, O); each gene had 13 three target sequences. Mouse genomic sequence data were obtained from 14 GRCm38/mm10 via the UCSC Genome Browser (Rhead et al., 2010) 15 (http://genome.ucsc.edu/). Colored letters (blue, orange, and green) show the 16 20-base target sequences. The target sequences were designed on the sense (+) or 17 antisense (-) strand of genomic DNA. (J-N, P, and R-V) The genotype of KO mice for the plasma membrane Ca²⁺ ATPase

18

19 and calcium/calmodulin-dependent protein kinase family [Atp2b2 (J), Atp2b3 (K-M),

20 Atp2b4 (N), Camk2a (P and R), Camk2b (S and T), Camk2d (U) and Camk2g (V)];

21 The relative amount of intact DNA for each target sequence was measured by

22 quantitative PCR (gPCR). The genomic DNA was purified from the tail of each mouse.

23 The relative amount of intact DNA for each target sequence was scaled so that the

24 level in a wild-type mouse was defined as 100%.

(Q) Genotyping by sequencing was done for two *Camk2a* KO mice (set 1). The
 sequences of mutant alleles in each mouse are shown. The target sites are colored
 and underlined. The mutations are labeled in red. The numbers of each detected
 allele are put in parentheses.

1 Supplemental Movies

- 2 Movie S1. Whole-brain Arc-dVenus image of control mice, related to Figure 5A.
- 3 Representative reconstituted 3D images of the Arc-dVenus mouse brain at CT4 and
- 4 CT16 without the chronic administration of MK-801. Green: Arc-dVenus signals.
- 5 Gray: TOPRO3 signals. CT: Circadian time.
- 6

- 1 Movie S2. Whole-brain Arc-dVenus image of MK-801 treated mice, related to
- 2 Figure 5A.
- 3 Representative reconstituted 3D images of the Arc-dVenus mouse brain at CT4 and
- 4 CT16 with the chronic administration of MK-801. Green: Arc-dVenus signals. Gray:
- 5 TOPRO3 signals. CT: Circadian time.

Movie S3. Brain-region analysis of hyper-activated cells in MK-801 treated and untreated mice, related to Figures 5C-5E.

Representative 3D-reconstituted brain images of the Arc-dVenus signals extracted
from cells detected as hyper-activated and located in regions belonging to the same
heatmap cluster (shown in pink, yellow, and blue) at CT4 and CT16 with and without
the chronic administration of MK-801. Gray indicates TOPRO3 signals. CT: Circadian
time.

Movie S4. Cortex-layer analysis of hyper-activated cells in MK-801 treated and untreated mice, related to Figures 6A-6C.

- 3 3D-reconstituted brain images of the Arc-dVenus signals extracted from cells
- 4 detected as hyper-activated and located in regions belonging to the same layer
- 5 (shown in cyan (layer 1), magenta (layer 2-3), and yellow (layer 4-6)) at CT4 with and
- 6 without the chronic administration of MK-801. Grey indicates TOPRO3 signals. CT:
- 7 Circadian time.

1 Supplemental Tables

2 Table S1. Parameter values in the averaged-neuron model, related to

3 Simulations in Experimental procedures.

4 Values from C to T_{GABA} were based on previous studies (Bazhenov et al., 2002; Chen 5 et al., 2012; Compte et al., 2003; Hill and Tononi, 2005; Sanchez-Vives et al., 6 2010; Timofeev et al., 2000). The revised values are indicated by asterisks. The area 7 of neuron (A) was revised because we did not divide the model neuron into dendritic and synaptic parts. The coefficient of Ca²⁺-entry (α_{Ca}) is estimated under the 8 assumption that the average volume of a single neuron is ~10 pl. The increase in 9 intracellular Ca²⁺ concentration caused by Ca²⁺ currents was determined as follows: 1 10 nA Ca²⁺ current for 1 ms causes 1 nA × 1 ms / ~10 pl = 1 p C/ ~10 pl = ~ 0.5 /10 F 11 mol/L = $\sim 0.5 \,\mu$ M increase in the intracellular Ca²⁺ concentration, where F = $\sim 0.96 \times$ 12 10^5 is the Faraday constant. The values from g_L to τ_{Ca} are an example of parameter 13 14 sets that yield slow-wave-sleep firing patterns in the wild-type parameter search. This 15 parameter set was used in Figures 1B and 1C; Figure 1F (dash line); Figures **S1A-S1G**. In the parameter searches, 13 parameter values (g_L to τ_{Ca}) are randomly 16 17 searched.

С	Membrane capacitance	1 µF/cm ²
А	Area of neuron	0.02 mm ² *
VL	Leak reversal potential	-60.95 mV
V _{Na}	Sodium reversal potential	55 mV
Vκ	Potassium reversal potential	-100 mV
T _{hA}	Time-constant of <i>h</i> _A	15 ms
V _{Ca}	Ca ²⁺ reversal potential	120 mV
KD	Dissociation constant of Ca ²⁺ -dependent K ⁺	30 µM
	channels	
V _{AMPA}	APMA receptor reversal potential	0 mV

V _{NMDA}	NMDA receptor reversal potential 0 mV			
V _{GABA}	GABA _A receptor reversal potential -70 mV			
α _{Ca}	Coefficient of Ca ²⁺ -entry 0.5 µM/(nA ms) *			
T AMPA	Time-constant of s2 ms			
T _{SNMDA}	Time-constant of s_NMDA100 ms			
T _{XNMDA}	Time-constant of x_{NMDA} 2 ms			
T GABA	Time-constant of s _{GABA}	10 ms		
$g_{ m L}$	The conductance of the leak K^+ channels	0.03573 mS/cm ²		
g _{Na}	The conductance of the voltage-gated Na ⁺ 12.2438 mS/cm ²			
gк	The conductance of the voltage-gated K ⁺ 2.61868 mS/cm ² channels 2.61868 mS/cm ²			
g A	The conductance of the fast A-type K^+ 1.79259 mS/cm ² channels			
g кs	The conductance of the slowly inactivating K ⁺ 0.0350135 mS/cm ² channels			
g _{NaP}	The conductance of the persistent Na ⁺ channels	0.0717984 mS/cm ²		
g ar	The conductance of the inwardly rectifying K ⁺ channels	0.0166454 mS/cm ²		
g _{Ca}	The conductance of the voltage-gated Ca2+0.0256867 mS/cm2channels			
д кса	The conductance of the Ca^{2+} -dependent K ⁺ 2.34906 mS/cm ² channels			
g ampa	The conductance of the AMPA receptors 0.513425 µS			
g NMDA	The conductance of the NMDA receptors 0.00434132 µS			
<i>G</i> GABA	The conductance of the GABA _A receptors	0.00252916 µS		
<i>т</i> _{Са}	The time constant of Ca ²⁺ efflux	121.403 ms		

- 1 Table S2. Oligonucleotide sequences for the T7-gRNA templates, related to
- 2 Animals and sleep phenotyping in Experimental procedures.
- gRNAs were produced by the T7-gRNA templates of *Kcnn1*, *Kcnn2*, *Kcnn3*, *Kcnn4*, *Kcnt1*, *Kcnt2*, *Kcnu1*, *Cacna1a*, *Cacna1b*, *Cacna1c*, *Cacna1d*, *Cacna1e*, *Cacna1f*, *Cacna1g*, *Cacna1h*, *Cacna1i*, *Cacna1s*, *Atp2b1*, *Atp2b2*, *Atp2b3*, *Atp2b4*, *Camk2a*, *Camk2b*, *Camk2g*, *and Camk2d* which were made from oligonucleotides (Hokkaido
 System Science). The alternative target sequence for *Kcnn2*, *Kcnn3*, *Cacna1g*, *Cacna1h*, *Atp2b3*, *Camk2a*, *and Camk2b* (set 2 in Figures S2J and S2K; Figures
 S4K and S4L; Figures S7I and S7O) are shown as gRNA No. 4-6 in each gene.

- 1 Table S3. Primer sequences used in quantitative PCR (qPCR), related to
- 2 Animals and sleep phenotyping in Experimental procedures.
- 3 1st qPCR primers (Hokkaido System Science) were used in genotyping of knockout
- 4 mice Kcnma1, Kcnn1, Kcnn2, Kcnn3, Kcnn4, Kcnt1, Kcnt2, Kcnu1, Cacna1b,
- 5 Cacna1e, Cacna1f, Cacna1g, Cacna1h, Cacna1i, Atp2b2, Atp2b3, Atp2b4, Camk2a,
- 6 *Camk2b, Camk2g,* and *Camk2d*. The alternative 2nd qPCR primers were used in
- 7 genotyping of knockout mice of *Kcnn2*, *Atp2b3*, *Camk2a*, *Camk2b*, and *Camk2g*.

- 1 Table S4. Primer sequences used in cloning for genotyping, related to Animals
- 2 and sleep phenotyping in Experimental procedures.
- 3 Primer sequences used in cloning for genotyping of *Kcnn1*, *Kcnn2*, *Kcnt2*, *Cacna1h*,
- 4 and *Camk2a* (Hokkaido System Science).

Gene	gRNA No.	Primer	Sequence (5' to 3')
Kcnn1	1	Forward	GATGACAGTGGACAGGCTGATGAGGC
		Reverse	AGGTCCATTCAGAAGCTCTGCTGCGG
	2 and 3	Forward	CTTCAGCTGGACCTGGTGGCTCTGAGC
		Reverse	GCTGTTCTTGGTGGACAATGGTGCCG
Kcnn2	1	Forward	GGATAACCTGTCCCTGCTGCTCC
		Reverse	GGGACACTTAAGACCTGTTAGACAA
	2 and 3	Forward	CCTAAAGTACTTAAACTCCGGAGCA
		Reverse	CATGTTGATAGAAGCAGTGAACCAA
	1	Forward	CACCACCTACCCTCTTTCTAGGCACCGC
		Reverse	CCCAGTGGCAAGCAAACATCTCGTCGAG
Kont2	2	Forward	GAGCTGTTTTTAGGATTGGGAAACAGGGGC
NCI112		Reverse	GCATGACCATCACCCAGTGACTACACCAC
	3	Forward	GTGGCACTGTGGCTGACTGAGCCTGAC
		Reverse	GTACCGCCATCCCACCCATCTCTGCC
	1	Forward	GACCCTCACTTAGGCTACCAGGCTC
Cacna1h		Reverse	CTGGGATTCCCAAGCATACCCAGCC
	2	Forward	CTATCCAGTGTGCGTGTTGCGGGACTC
		Reverse	GCAGGATGCCCACCAGCTGGCCAC
	3	Forward	GCCTCACAGGACTAGGCCGAGCCTC
		Reverse	GCATAGAGGCCCTTGCAGTGCCTC
Comble	1	Forward	GTGACAGCCACTAAATCAGGCAACGTG
		Reverse	GAGGTCAAAGACCTGTGGAGAACCAGGGC
Carrikza	2 and 3	Forward	CCATTATTTGGGTTACACTAGGGGA
		Reverse	AGAATTCCAGCAAAATCCAAAGGAG

- 1 Table S5. FISH primers, related to Fluorescence in situ hybridization in
- 2 Experimental procedures.
- 3 FISH probes for Gad1, Gad2, Vglut1, Vglut2, c-Fos, and Nr2b were produced by in
- 4 *vitro* transcription with T3-probe PCR fragments. These probes were used as mixes;
- 5 c-Fos-1 and 2 (*c-Fos* probes), Nr2b-1, 2 and 3 (*Nr2b* probes), Vglut1-1, Vglut2-1, 2
- 6 and 3 (*Vglut1/2* probes), or Gad1-1, 2, Gad2-1, 2 and 3 (*Gad1/2* probes).

Gene	No.	Primer	Sequence
Gad1	1	Forward	GCCTGAAGATCTGTGGCTTC
		Reverse	AATTAACCCTCACTAAAGGGTAGCCCTTTTGTTTGGCATC
		Forward	CACAAACTCAGCGGCATAGA
	2	Reverse	AATTAACCCTCACTAAAGGGGGACGAGCAACATGCTATGG
Gad2	1	Forward	GGGATGTCAACTACGCGTTT
		Reverse	AATTAACCCTCACTAAAGGGTGCATCAGTCCCTCCTCT
	2	Forward	CTCCAATCCCCTTCTTCTCC
	2	Reverse	AATTAACCCTCACTAAAGGGTGTGCATCCTTTGTCCATGT
	2	Forward	AGCCAGGGCTACACAGAGAA
	3	Reverse	AATTAACCCTCACTAAAGGGTTTGCTAGGGCTGATTTGGT
Slc17a7	1	Forward	CTGGCAGTGACGAAAGTGAA
(Vglut1)	1	Reverse	AATTAACCCTCACTAAAGGGACACAACAAATGGCCACTGA
	1	Forward	TAGCTTCCTCTGTCCGTGGT
		Reverse	AATTAACCCTCACTAAAGGGGGGCCAAAATCCTTTGTTTT
Slc17a6	2	Forward	CCACCAAATCTTACGGTGCT
(Vglut2)		Reverse	AATTAACCCTCACTAAAGGGGGGGGGCATACCCCTCCCTTA
	3	Forward	CTCCCCCATTCACTACCTGA
		Reverse	AATTAACCCTCACTAAAGGGGGTCAGGAGTGGTTTGCATT
	1	Forward	AGCGAGCAACTGAGAAGACTG
c-Fos		Reverse	AATTAACCCTCACTAAAGGGATCTCCTCTGGGAAGCCAAG
	2	Forward	CCAGTCAAGAGCATCAGCAA
		Reverse	AATTAACCCTCACTAAAGGGCATTCAGACCACCTCGACAA
Grin2b (Nr2b)	1	Forward	CGGGCTACTAACCTCACATGC
		Reverse	AATTAACCCTCACTAAAGGGCCAAACTGGAAGAACATGGAGGAC
	2	Forward	ACTTCGTGAACAAGATCCGCAG
		Reverse	AATTAACCCTCACTAAAGGGCATGCAGGTCCCACTGAGAG

0	Forward	ATCTCTTCTATTGGCAGTTCCGAC
3	Reverse	AATTAACCCTCACTAAAGGGGGACTGTCCTTATATTGCTGCTTCCTC
		*T3 promoter sequence is underlined.

Table S6. Spatio-temporal profiling of the estimated number of hyper-activated cells in each brain region, related to Figure 5B

It contains temporal variation in the estimated number of hyper-activated cells for 826
different brain regions, ordered alphabetically by their abbreviation. For each region,
we give: abbreviation, full name, cluster (with "N/A" for regions filtered out before the
clustering due to low signals), normalized 8-point data for clustering and all 20-brains
data.

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- 11





Figure S1. F. Tatsuki et al.


В		Kcnn1		
chr8(-) 7	0,855,000	70,845,000		
		1-1-1-1-1	Kcnn1	
ccctctttgaaaa Kcnn1 target	agcgtaaa (+) sequence-1	gttgaaggtgacgcggttaa (-) Kcnn1 target sequence-2	agaccaacaacacagtgccg (- Kcnn1 target sequence-3	

D		Kcnn3 (se	t 1)	
chr3(+) 89,55	50,000	89,600,000	89,650,00	D
1	erii eefenne	· k		Kcnn3
gacacttggggtcttcal Kcnn3 target seque	tcc (-) go ence-1 K	ctcctgcaaatacagcggt (cnn3 target sequence	+) gaggatacgo -2 Kcnn3 targo	ctcgtaggtca (-) et sequence-3
F		Kcnt1		
chr2(+)	25,880,0	00	25,910,00	0
**************************************	11.1			Kcnt1
gccacttacctatcgtc	qtt (-) g	aaatcttccacatgaagcc	(-) gtgatgtgaco	cccaagatc (+)
Kcnt1 target seque	ence-1 K	Cont1 target sequence	-2 Kcnt1 targe	et sequence-3
н		Kcnu1		
chr8(+) 25,86	0,000	25,890,000	25,920,000	
			+++-+++	Kcnu1
gtcatgtacaatcgaaa Kcnu1 target seque	tcc (+) ga	aagttggaaatacgtcaag cnu1 target sequence	(+) gaatcgagg a-2 Kcnu1 targ	ctaatcctttg (+) et sequence-3

Morning Night ** Kcnma1 ** Kcnn1 Kcnn2 (set 1) *** *** Kcnn3 (set 1) ** *** Kcnn4 * Kcnt1 -* • Kcnt2 Kcnu1 . * 600 200 300 400 400 500 100 300 Sleep duration (min) L n.s 0.04 0.03 0.02 L 0.03 م[§] 0.02 0.01 0.01 0.00 0.00 WT Kcnn2 WT Kcnn2

	Kc	nn2 (set 2)		
45,600,	000	45,650,00	00	
1			++++++	Kcnn2
ctaagc (+)	ctaacaaco	tggcgctctac (+)	gtgctatccatccc	atacct (+)
	45,600,	45,600,000	45,600,000 45,650,00 45,650,00 tage (+) claacaacclggcgctctac (+)	45,600,000 45,650,000 45,650,000 time (core 2) 45,650,0000 45,650,0000 45,650,0000 45,650,0000 45,650,0000 45,650,0000 45,650,0000 45,650,0000 45,650,000000000000000000000000000000000

κ		Kcnn3 (set 2)
chr3(+)	89,550,000 I	89,600,000	89,650,000
ctctcggag Kcnn3 tar	aaaccttatcg (+) get sequence-4	gcgtatcctctacatcagcc (+) Kcnn3 target sequence-5	gagcgtcttcatgacgaatc (-) Kcnn3 target sequence-6





В

Α



























wt

С

Figure S3. F. Tatsuki et al.

Α	Cacna1a	
chr8(+) 84,450,000	84,550,000	
		Cacna1a
1	1*	++++++++++
gacacggaaccatacttcat (+)	ggaccaaatgtcagccgtac (+)	gaactgagtgatgccgttgt (-)







ggcaaagatgaagtcgtcga (-) gcaataccacctgtgtcaac (+) gggaatcatgatcgctatcc (+) Cacna tg target sequence-1 Cacna tg target sequence-2 Cacna tg target sequence-3



Cacna1b target sequ Cacna1b target sequence-2 Cacna1b target se

Cacna1e

154,500,000

Cacna 1e

Е

chr1(-) 154,700,000

Ν



attat Cacna1c target sequence-1 na1c target sequ ce-2 Cacna1c target seque

chrX(+)	7,610,000	7,620,000	7,630,000	
		· · · · · · · · · · · · · · · · · · ·		acna1f

gtgaaacagttctgccggac (-) gttcatacctccgtaatggc (+) gtccacgtggggttgaagt (-) Cacna 1e target sequence-1 Cacna 1e target sequence-2 Cacna 1e target sequence-3 a1/target sequence-1 Cacna1f target sequence-2 Cacna1f target sequence-3



gttcggccagaaatgctacc (+) ggctccggcgtttaaccttt (-) gg Cacna th target sequence-1 Cacna th target sequence-2 Can na1h target sequence-3



J Cacna1s chr1(+) 136,070,000 136,090,000 136,110,000 Cacna1s 11 11 11 11 11 11 11 gatgttcccgatggtgcgaa (-) gggctactactacatataca (+) gtcctcctatttgagtcta (-) Cacna 1s target sequence-1 Cacna 1s target sequence-2 Cacna 1s target sequence-3





galalgggtatagaccitig (-)
genggalaccitig (-)
g





Ο Cacna1f 120 100 80 60 40 20 0 wt 1 2 3 4 5 6 8 9 10 7















Figure S5. F. Tatsuki et al.



Figure S6. F. Tatsuki et al.

