Activity- and sleep-dependent regulation of tonic inhibition by Shisa7

Graphical abstract

Highlights

- Shisa7 is critical for the regulation of tonic inhibition in hippocampal neurons
- PKA phosphorylates Shisa7 to modulate α5-GABAR exocytosis and tonic inhibition
- Shisa7 is important for activity-dependent regulation of tonic inhibition
- The sleep/wake cycle regulates tonic inhibition in a Shisa7-dependent manner

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In brief

Wu et al. discover a critical role of Shisa7 in the regulation of tonic inhibition in hippocampal neurons and find that PKA phosphorylates Shisa7 to modulate activity-dependent regulation of tonic inhibition. They also show that Shisa7 is involved in tonic inhibition regulation over the daily sleep/wake cycle.
Activity- and sleep-dependent regulation of tonic inhibition by Shisa7

Kunwei Wu, Wenyan Han, Qingjun Tian, Yan Li, and Wei Lu

INTRODUCTION

Tonic inhibition mediated by extrasynaptic \(\gamma\)-aminobutyric acid type A receptors (GABA\(_ARs\)) critically regulates neuronal excitability and brain function. However, the mechanisms regulating tonic inhibition remain poorly understood. Here, we report that Shisa7 is critical for tonic inhibition regulation in hippocampal neurons. In juvenile Shisa7 knockout (KO) mice, \(\alpha5\)-GABA\(_AR\)-mediated tonic currents are significantly reduced. Mechanistically, Shisa7 is crucial for \(\alpha5\)-GABA\(_AR\) exocytosis. Additionally, Shisa7 regulation of tonic inhibition requires protein kinase A (PKA) that phosphorylates Shisa7 serine 405 (S405). Importantly, tonic inhibition undergoes activity-dependent regulation, and Shisa7 is required for homeostatic potentiation of tonic inhibition. Interestingly, in young adult Shisa7 KOs, basal tonic inhibition in hippocampal neurons is unaltered, largely due to the diminished \(\alpha5\)-GABA\(_AR\) component of tonic inhibition. However, at this stage, tonic inhibition oscillates during the daily sleep/wake cycle, a process requiring Shisa7. Together, these data demonstrate that intricate signaling mechanisms regulate tonic inhibition at different developmental stages and reveal a molecular link between sleep and tonic inhibition.

SUMMARY

Tonic inhibition mediated by extrasynaptic \(\gamma\)-aminobutyric acid type A receptors (GABA\(_ARs\)) critically regulates neuronal excitability and brain function. However, the mechanisms regulating tonic inhibition remain poorly understood. Here, we report that Shisa7 is critical for tonic inhibition regulation in hippocampal neurons. In juvenile Shisa7 knockout (KO) mice, \(\alpha5\)-GABA\(_AR\)-mediated tonic currents are significantly reduced. Mechanistically, Shisa7 is crucial for \(\alpha5\)-GABA\(_AR\) exocytosis. Additionally, Shisa7 regulation of tonic inhibition requires protein kinase A (PKA) that phosphorylates Shisa7 serine 405 (S405). Importantly, tonic inhibition undergoes activity-dependent regulation, and Shisa7 is required for homeostatic potentiation of tonic inhibition. Interestingly, in young adult Shisa7 KOs, basal tonic inhibition in hippocampal neurons is unaltered, largely due to the diminished \(\alpha5\)-GABA\(_AR\) component of tonic inhibition. However, at this stage, tonic inhibition oscillates during the daily sleep/wake cycle, a process requiring Shisa7. Together, these data demonstrate that intricate signaling mechanisms regulate tonic inhibition at different developmental stages and reveal a molecular link between sleep and tonic inhibition.

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Glykys and Mody, 2007; Jacob, 2019; Martin et al., 2009; Mohamad and Has, 2019). Indeed, rare missense mutations in the α5 subunit have been identified in numerous patients with neurological and psychiatric disorders (Butler et al., 2018; Hernandez et al., 2019; Hodges et al., 2014). Furthermore, preclinical studies demonstrate that allosteric modulators that target α5-GABA<sub>A</sub>Rs could be potentially therapeutic candidates for use in neurodevelopmental and neuropsychiatric disorders (Jacob, 2019; Mohamad and Has, 2019). However, despite the importance of α5-GABA<sub>A</sub>R-mediated tonic inhibition in neuronal physiology and animal behaviors, the molecular mechanisms underlying trafficking and activity-dependent regulation of α5-GABA<sub>A</sub>Rs and tonic inhibition currently remain poorly understood.

We recently identified a GABA<sub>A</sub>R auxiliary subunit, Shisa7, which is a single-pass transmembrane protein that interacts with either α1- or α2-GABA<sub>A</sub>Rs and thereby regulates inhibitory synaptic transmission (Han et al., 2019). Here, we report that Shisa7 also plays a critical role in the regulation of tonic inhibition in hippocampal CA1 neurons. In the juvenile Shisa7 knockout (KO) mice, both α5-GABA<sub>A</sub>R exocytosis and tonic inhibition are significantly reduced. Furthermore, Shisa7-dependent regulation of tonic inhibition requires protein kinase A (PKA) that phosphorylates Shisa7 at serine 405 (S405). Importantly, tonic inhibition undergoes Shisa7-dependent homeostatic upregulation, revealing a previously uncharacterized form of neuronal plasticity. At the behavioral level in young adult mice, the daily sleep/wake oscillation regulates tonic inhibition in a Shisa7-dependent manner, a process requiring the modulation at Shisa7 S405. Together, these data characterize critical molecular pathways regulating tonic inhibition and demonstrate how behavioral processes engage neuronal molecular signaling to modulate neural inhibition in the brain.

RESULTS

Tonic inhibitory currents are reduced in Shisa7 KO hippocampal neurons

To measure tonic inhibition, we performed whole-cell voltage-clamp recordings in hippocampal cultures. Application of bicuculline (BIC; 20 µM), a competitive antagonist of GABA<sub>A</sub>Rs, to cultured neurons readily reduced baseline holding currents, revealing tonic currents mediated by extrasynaptic GABA<sub>A</sub>Rs (Figure 1A). To account for the effect of cell size on tonic currents, we normalized tonic currents by the cell membrane capacitance, which did not significantly differ between wild-type (WT) and Shisa7 KO neurons (Figure S1). We found that compared to WT neurons, tonic currents in Shisa7 KO hippocampal neurons were significantly reduced (Figures 1A and S1A). In addition to cultured neurons, we measured tonic currents in acute hippocampal slices prepared from postnatal day 16 (P16) to P21 juvenile WT or Shisa7 KO mice. We found that GABA<sub>A</sub>R-mediated tonic currents were substantially reduced in hippocampal CA1 neurons from Shisa7 KO mice (Figures 1B and S1B). Similarly, tonic currents were smaller in dentate gyrus (DG) granule cells in Shisa7 KO mice as compared to WT mice (Figures 1C and S1C), showing the importance of Shisa7 in the regulation of tonic inhibition in different hippocampal cell types. Together, these data demonstrate a critical role of Shisa7 in the regulation of tonic inhibition in hippocampal neurons.

Previous work has shown that extrasynaptic α5-GABA<sub>A</sub>Rs mediate the majority of tonic inhibition in hippocampal CA1 neurons (Caraiscos et al., 2004; Glykys et al., 2008). Thus, we explored the role of Shisa7 regulation in both α5-GABA<sub>A</sub>Rs-dependent and independent components of tonic currents in these neurons. Similar to previous reports (Caraiscos et al., 2004; Glykys et al., 2008), application of L-655,708, a potent α5-GABA<sub>A</sub>R inverse agonist (Quirk et al., 1996), strongly reduced tonic inhibitory currents by ~70% in WT CA1 neurons (Figure 1D). Interestingly, in Shisa7 KO CA1 neurons, L-655,708-sensitive components of tonic currents were significantly reduced, while there was little change in L-655,708-insensitive tonic currents (Figure 1D). In hippocampal CA1 neurons, L-655,708-insensitive tonic inhibitory currents are largely mediated by δ-GABA<sub>A</sub>Rs (Glykys et al., 2008). THIP (4,5,6,7-tetrahydroxyisoxazol [4,5-c]pyridine-3-ol), a GABA<sub>A</sub>R agonist with a preference for δ-GABA<sub>A</sub>Rs (Brown et al., 2002), evoked a similar amount of currents in WT and Shisa7 KO CA1 neurons (Figure 1E), showing that Shisa7 KO did not alter δ-GABA<sub>A</sub>R-mediated currents. Collectively, these data indicate that Shisa7 KO reduces tonic inhibition mediated by α5-GABA<sub>A</sub>R, but not δ-GABA<sub>A</sub>Rs, in hippocampal CA1 neurons.

Shisa7 interacts with α5-GABA<sub>A</sub>Rs and regulates receptor trafficking

We have recently shown that Shisa7 can be co-immunoprecipitated with α1- and α2-GABA<sub>A</sub>Rs and promotes trafficking of these receptors to the cell surface and inhibitory synapses (Han et al., 2019). We thus examined whether Shisa7 could also interact with α5-GABA<sub>A</sub>Rs. In HEK293T cells, we found that GFP-tagged α5 (α5-GFP) was co-immunoprecipitated with Flag-tagged Shisa7 (Flag-Shisa7) from cells co-transfected with both constructs, but not from those transfected with either plasmid alone (Figures 2A and S2A). Similarly, in detergent-solubilized mouse hippocampal lysates, the α5 subunit was detected in the Shisa7 immunoprecipitates (Figures 2B and S2B). Interestingly, neither α4 nor δ subunits were co-immunoprecipitated with Shisa7 (Figure 2B), showing that Shisa7 is associated with α5-, but not α4- or δ-, GABA<sub>A</sub>Rs in the hippocampus.

We then examined whether Shisa7 regulated α5-GABA<sub>A</sub>R trafficking. In HEK293T cells, co-expression of Shisa7 with α5β3γ2 receptors significantly increased surface levels, total levels, and surface to total ratio of α5 expression (Figure 2C). Similarly, GABA-evoked α5β3γ2-mediated whole-cell currents were significantly increased in HEK293T cells co-expressing Shisa7 compared to cells co-expressing GFP (Figure 2D). Thus, Shisa7 promotes α5-GABA<sub>A</sub>R trafficking to the cell surface in heterologous cells. In hippocampal cultures, we found that surface levels of α5 were significantly reduced in Shisa7 KO neurons compared to WT neurons (Figure 2E). In addition, total α5 expression was reduced in Shisa7 KO neurons (Figure 2E), suggesting that Shisa7 may play a role in the regulation of α5 expression and/or stability in hippocampal neurons. Furthermore, the surface to total ratio of α5 expression was significantly decreased in Shisa7 KO neurons (Figure 2E), indicating that α5-GABA<sub>A</sub>Rs trafficking to the neuronal surface is impaired in neurons lacking Shisa7. Collectively, these data
demonstrate that Shisa7 is important for α5-GABAAR trafficking to the cell surface.

The reduction of the surface levels of α5 in Shisa7 KO neurons might involve increased endocytosis of surface α5 or reduced exocytosis of intracellular α5. To differentiate between these possible scenarios, we first performed antibody-feeding experiments to label surface and internalized endogenous α5 in live hippocampal cultures and examined α5 endocytosis in WT and Shisa7 KO neurons. We found that Shisa7 KO did not significantly change α5 internalization (Figure S2D). We then combined fluorescence recovery after photobleaching (FRAP) with fluorescence loss in photobleaching (FLIP) to investigate exocytosis of superrecluptic pHluorin-tagged α5 (SEP-α5) to measure the receptor exocytosis. In this experiment, repetitive photobleaching occurred at dendritic regions bilateral to the central FRAP area, thus excluding laterally diffusing SEP-α5 to the central area and allowing the measurement of newly exocytosed SEP-α5 (Figures 2F and 2G). We found that while SEP-α5 expressed in WT neurons showed a substantial recovery within 5 min after photobleaching, much smaller fluorescence recovery of SEP-α5 was observed in Shisa7 KO neurons (Figures 2F and 2G), indicating that Shisa7 KO reduces α5 exocytosis.

Protein kinases regulate tonic inhibition

What are the molecular mechanisms underlying the regulation of tonic inhibition by Shisa7? Protein phosphorylation has been shown to play critical roles in regulating dynamic trafficking of neurotransmitter receptors (Connelly et al., 2013; Lu and Roche, 2012; Nakamura et al., 2015). To explore the potential role of protein kinases in the regulation of tonic inhibition, we performed pharmacological inhibition assays in hippocampal cultures and measured tonic inhibitory currents. We found that pharmacological inhibition of PKA, protein kinase C (PKC), or CaMKII led to a significant reduction of tonic currents in WT neurons (Figure 3A), showing that tonic inhibition is sensitive to activities of a variety of protein kinases. Strikingly, in Shisa7 KO neurons, although blockade of CaMKII activity still...
decreased tonic inhibitory currents, the effects of inhibition of PKA or PKC activities on tonic currents were abolished (Figure 3A), indicating that those kinases modulate tonic inhibition through Shisa7.

PKA phosphorylates Shisa7 to regulate α5 exocytosis and tonic inhibition

Given that either PKA or PKC requires Shisa7 to regulate tonic inhibition, we hypothesized that Shisa7 was a phosphorylation substrate of PKA or PKC. To this end, we purified glutathione S-transferase (GST) fusion to Shisa7 C-terminal (C-tail) fragments and performed an in vitro phosphorylation assay with recombinant PKA or PKC. We then utilized liquid chromatograph coupled to tandem mass spectrometry (LC-MS/MS) to identify phosphorylated residues. We found several serine or threonine residues on the C-tail of Shisa7 that were substrates of PKA and/or PKC in vitro (Figure 3B). To further evaluate whether these phosphorylation events occurred in vivo, we performed an immunoprecipitation assay to pulldown endogenous Shisa7 from mouse hippocampal lysates and used LC-MS/MS to detect phosphorylated residues of neuronal Shisa7. We identified two phosphorylated serine residues (S405 and S430) in neuronal Shisa7. We found several serine or threonine phosphorylated residues. We found several serine or threonine phosphorylated residues. We found several serine or threonine phosphorylated residues.

In addition to the two above-mentioned serine residues that were substrates of PKA (Figures 3B and 3C), showing that these two residues can be phosphorylated both in vitro and in vivo.

In hippocampal cultures prepared from Shisa7 KO mice, expression of WT Shisa7 significantly increased tonic inhibition currents (Figures 4A–4C). In contrast, expression of the Shisa7 mutant lacking the majority of C-tail (Shisa7 ΔC) did not rescue the tonic current deficit (Figures 4A–4C), showing the importance of Shisa7 C-tail in regulating tonic inhibition.
We then generated point mutation at S306, S405, or S430, mutating these serine residues to alanine (A), respectively, and expressed them individually in Shisa7 KO hippocampal neurons. Intriguingly, while both Shisa7 S306A and Shisa7 S430A restored tonic current deficits in Shisa7 KO neurons, Shisa7 S405A failed to rescue the reduced tonic currents (Figures 4A–4C), demonstrating that Shisa7 S405 plays a critical role in the regulation of tonic inhibition. Similar results were obtained in overexpression experiments (Figure S3). Specifically, while overexpression of Shisa7 or Shisa7 phosphomimetic (S405D) mutant substantially increased tonic inhibitory currents in WT hippocampal cultures, tonic inhibition was significantly decreased in neurons expressing Shisa7 ΔC or Shisa7 S405A (Figure S3).

What are the mechanisms for the inability of Shisa7 S405A to rescue tonic current deficits in Shisa7 KO neurons? We found that transfection of WT Shisa7 back into Shisa7 KO neurons significantly increased expression levels of surface α5 (Figure 4D). In contrast, expression of Shisa7 S405A did not significantly alter surface α5 expression (Figure 4D), suggesting that S405 is important in the regulation of surface abundance of α5-GABARs. FRAP-FLIP experiments in live hippocampal neurons further showed that Shisa7, but not Shisa7 S405A, restored the reduced exocytosis of SEP-α5 in Shisa7 KO neurons (Figures 4E and 4F), showing that S405 is crucial for α5-GABAAR forward trafficking to the neuronal surface. Thus, Shisa7 S405 controls α5-GABAAR exocytosis, which in turn regulates tonic inhibition.

**Homeostatic potentiation of tonic inhibition requires Shisa7 S405**

Homeostatic plasticity plays a critical role in regulating synaptic strength and neuronal excitability. Both excitatory and inhibitory synapses have been shown to be capable of undergoing homeostatic regulation in response to chronic changes of...
neuronal network activity (Hartman et al., 2006; Kilman et al., 2002; Turrigiano et al., 1998; Turrigiano and Nelson, 2004). However, it remains unclear whether tonic inhibition can also undergo homeostatic plasticity induced by chronic activity manipulation. To this end, we applied tetrodotoxin (TTX) to block action potentials or used a GABA AR competitive antagonist, BIC, to chronically reduce or increase neuronal activity, respectively, in hippocampal cultures and then measured tonic inhibition. We found that in WT neuronal cultures, TTX strongly decreased and BIC significantly increased tonic inhibitory currents (Figure 5A), demonstrating bidirectional plasticity of tonic inhibitory currents in response to chronic change of neuronal activity. Strikingly, in Shisa7 KO hippocampal cultures, while TTX treatment substantially depressed tonic currents, BIC did not significantly alter tonic inhibition (Figure 5A), indicating that Shisa7 is critical for homeostatic potentiation of tonic inhibition in hippocampal neurons. Application of L-655,708 to block α5-GABAARs revealed that BIC-induced potentiation of tonic currents was largely mediated by α5-GABAARs (Figure S4). In addition to measuring tonic currents, we performed immunocytochemical assays to determine the abundance of surface α5-GABAARs on hippocampal neurons treated with TTX or BIC. We found that TTX treatment substantially reduced surface abundance of α5-GABAARs in both WT and Shisa7 KO cultures (Figure 5B). In contrast, although BIC treatment increased surface abundance of α5-GABAARs in WT cultures, it failed to induce upregulation of surface α5-GABAARs in Shisa7 KO cultures (Figure 5B).

To determine whether the requirement of Shisa7 in the homeostatic upregulation of tonic inhibition observed in neuronal cultures also occurs in vivo, we pharmacologically increased neuronal activity in live mice and then recorded tonic inhibitory currents in CA1 neurons in acute hippocampal slices (Figures 5C and 5E). We found that intraperitoneal (i.p.) injection of kainic acid (KA) increased tonic inhibition, which was blocked by APV, a NMDA receptor antagonist (Figure 5F). This result suggests that chronic KA treatment can induce homeostatic potentiation of tonic inhibition in vivo. Together, our findings provide a comprehensive understanding of the mechanisms underlying tonic inhibition and its homeostatic plasticity in hippocampal neurons.
acid (KA), which stimulates glutamate receptor activity, or pentylenetetrazol (PTZ), which noncompetitively inhibits GABA$_A$R activity, significantly increased tonic inhibition in WT mice (Figures 5D and 5F). Significantly, neither KA nor PTZ altered tonic inhibitory currents in CA1 neurons in hippocampal slices prepared from Shisa7 KO mice (Figures 5D and 5F). Taken together, Shisa7 is important for the homeostatic potentiation of tonic inhibition both in vitro and in vivo.

We have shown that PKA and Shisa7 S405 play a critical role in regulating tonic inhibition and a$_5$-GABA$_A$R exocytosis (Figures 3A, 4E, and 4F). Thus, it is possible that homeostatic potentiation of tonic inhibition induced by BIC treatment would be impaired in neurons treated with PKA inhibitors or expressing Shisa7 S405A mutant. To test this, we first treated WT hippocampal cultures with BIC and H89, a PKA inhibitor, and measured tonic inhibition (Figure 6A). We found that while BIC treatment on its own induced homeostatic potentiation of tonic inhibition both in vitro and in vivo, co-application of BIC and H89 abolished the upregulation of tonic currents (Figure 6B), indicating that BIC-induced increase of tonic inhibition requires PKA activity. We also expressed Shisa7 S405A mutant in hippocampal cultures and measured homeostatic upregulation of tonic inhibitory currents (Figure 6C). Similar to the data shown in Figure 5A, BIC treatment substantially increased tonic currents in control neurons (Figure 6D). However, in neurons expressing the Shisa7 S405A mutant, BIC treatment did not significantly induce homeostatic enhancement of tonic inhibition (Figure 6D). Thus, Shisa7 S405, a phosphorylation substrate of PKA, plays an important role in a$_5$-GABA$_A$R exocytosis as well as is critical for homeostatic upregulation of tonic inhibition.
Tonic inhibition changes over the daily sleep/wake cycle in a Shisa7-dependent manner

We have shown that tonic inhibition can be dynamically regulated by neuronal activity and that Shisa7 S405 plays a critical role in the regulation of homeostatic plasticity of tonic inhibition induced by chronic activity manipulation. We thus wondered whether homeostatic adaptation of tonic inhibition could be driven by behavioral states in vivo, such as sleep, as a number of homeostatic processes in the brain have been reported to be associated with the sleep/wake cycle (Cirelli, 2017; Tononi and Cirelli, 2014). To this end, we employed a PiezoSleep mouse behavioral tracking system to monitor sleep/wake cycles in young adult mice (6–8 weeks old) (Figure S5) and performed electrophysiological recordings to measure tonic inhibitory currents in hippocampal CA1 neurons in mice at sleep or wake states (Figures 7A and S5). We noticed that tonic currents in WT young adult mice were much smaller than those recorded in P16–P21 juvenile mice and that there was no significant difference of tonic inhibition in hippocampal CA1 neurons between young adult WT and Shisa7 KO mice (Figures 1B, 7D, and S6C). Application of L655,708, the α5-GABAAR inverse agonist, revealed that the L655,708-sensitive, α5-GABAAR-mediated component of tonic currents in WT CA1 neurons was substantially reduced in young adult mice (6–8 weeks old), whereas the L655,708-insensitive portion did not change compared with juvenile mice between 2 and 3 weeks old (Figures S6D–S6F). Thus, in young adult mice, there was no significant difference of tonic inhibition between WT and Shisa7 KO CA1 neurons, largely due to decreased α5-GABAAR-mediated tonic currents in WT neurons.

Figure 6. Shisa7 S405 is required for homeostatic potentiation of tonic inhibition

(A) Experimental design. WT hippocampal neurons were treated with BIC (40 μM), H89 (1 μM), or both for 48 h and then recorded for tonic currents.

(B) H89 abolished BIC-induced potentiation of tonic currents in WT hippocampal neurons (n = 9–11 for each group, one-way ANOVA, F = 13.72, p < 0.0001 with Tukey’s multiple comparisons test; Ctrl versus Ctrl + BIC, p = 0.0084; Ctrl versus H89, p = 0.0213).

(C) Experimental design. WT hippocampal neurons were transfected with Shisa7 S405A at DIV16 and then treated with BIC (40 μM) for 48 h before recording.

(D) Expression of Shisa7 S405A blocked BIC-induced potentiation of tonic currents in WT hippocampal neurons (n = 6–9 for each group, one-way ANOVA, F = 20.30, p < 0.0001 with Tukey’s multiple comparisons test; Ctrl versus Ctrl + BIC, p = 0.0061; Ctrl versus Shisa7 S306A, p = 0.0212).

*p < 0.05 and **p < 0.01. All data are presented as mean ± SEM.

We found that in young adult WT mice, tonic inhibitory currents in hippocampal CA1 neurons changed over the 24-h sleep/wake cycle. Indeed, tonic inhibition was significantly higher in the animals that were awake compared to those asleep (Figure 7B). Strikingly, in Shisa7 KO CA1 neurons, there was no significant change of tonic currents over the daily sleep/wake cycle, and the tonic currents in Shisa7 KO neurons in either sleep or wake states were similar to those in WT mice in the sleep state (Figure 7B). Thus, Shisa7 KO disrupts the increase of tonic inhibition associated with the wake phase.

We also employed sleep deprivation (SD) to manipulate the daily sleep/wake cycle and measured the impact of sleep loss on tonic inhibition (Figure 7C). We found that a 6-h SD increased tonic currents in hippocampal CA1 neurons in WT young adult mice, but not in Shisa7 KO mice (Figure 7D). Thus, acute loss of sleep increases tonic inhibition in hippocampal CA1 neurons. Significantly, the L655,708-sensitive, α5-GABAAR-mediated component, but not the L655,708-insensitive component, of tonic currents was potentiated by SD in WT, but not Shisa7 KO, mice (Figures S6A and S6B), highlighting that SD-induced increases in tonic inhibition are α5 specific.

We have shown that Shisa7 S405 is critical for α5-GABAAR exocytosis and homeostatic upregulation of tonic inhibition in response to chronically elevated activity (Figures 4 and 6D). Therefore, we examined the role of Shisa7 S405 in SD-induced enhancement of tonic inhibition. MS analysis showed that SD increased expression levels of phosphorylated Shisa7 at S405 in the hippocampus (Figure S7). We then recorded tonic inhibitory currents in hippocampal CA1 neurons prepared from young adult mice that were in utero electroporated with Shisa7 S405A mutant (Figures 7E and 7F). We found that overexpression of Shisa7 S405A did not change tonic inhibition (Figure 7G), consistent with the data that the basal tonic inhibition in young adult mice does not depend on Shisa7 (Figure 7D). However,
SD-induced potentiation of tonic inhibition was abolished in neurons expressing Shisa7 S405A (Figure 7G), indicating that Shisa7 S405 is required for the new insertion of α5-GABAARs to the plasma membrane and, subsequently, upregulation of tonic inhibition associated with sleep loss.

**DISCUSSION**

Tonic inhibition mediated by extrasynaptic GABAARs plays a profound role in the regulation of neuronal excitability and brain function. In this study, we have uncovered a Shisa7-dependent molecular mechanism controlling the abundance of extrasynaptic GABAARs and tonic inhibition in hippocampus in juvenile mice. Importantly, tonic inhibition is bidirectionally regulated by neuronal activity, a process requiring Shisa7 phosphorylation. Furthermore, the sleep/wake cycle regulates tonic inhibition through a similar molecular pathway involving Shisa7 phosphorylation in young adult mice. Collectively, these data extend our recent work showing the importance of Shisa7 in regulating synaptic GABAARs and inhibitory transmission (Han et al., 2018; Han et al., 2021), provide mechanistic insights into modulation of tonic inhibition both in vitro and in vivo, and demonstrate a critical molecular link between sleep and tonic inhibition.

Shisa7 and α5-GABAAR-mediated tonic inhibition

In hippocampal CA1 pyramidal neurons, the majority of tonic inhibition is mediated by α5-GABAARs that are largely distributed at extrasynaptic membranes (Caraiscos et al., 2004; Glykys et al., 2008; Jacob, 2019). Thus, understanding the mechanisms controlling the abundance of α5-GABAARs at the cell surface will be critical for determining how tonic inhibition is dynamically regulated. Our data show that Shisa7 interacts with α5-GABAARs and promotes α5-GABAAR trafficking to the cell surface. Indeed, in heterologous cells, co-expression of Shisa7 promotes surface and total expression of α5-GABAARs in both heterologous cells and in neurons indicating that Shisa7 is critical for α5-GABAAR stability in cells. It is also worth noting that in hippocampal lysates, both α4 and δ subunits do not associate with Shisa7, demonstrating the specificity of Shisa7 with α5-GABAARs. Similarly, in Shisa7 KO hippocampal

![Experimental design. Sleep/wake patterns in young adult mice (P42–P56) were recorded based on the following criteria: “sleep mice” were asleep for at least 65% of the previous 4 h (≥60% per hour), whereas “wake mice” were awake for at least 75% of the previous 4 h (≥70% per hour).](image-url)

Figure 7. Tonic inhibition changes over the daily sleep/wake cycle in a Shisa7-dependent manner

(A) Experimental design. Sleep/wake patterns in young adult mice (P42–P56) were recorded based on the following criteria: “sleep mice” were asleep for at least 65% of the previous 4 h (≥60% per hour), whereas “wake mice” were awake for at least 75% of the previous 4 h (≥70% per hour).

(B) Tonic inhibition was increased in CA1 neurons in WT mice in wake, and Shisa7 KO abolished the increase of tonic inhibition associated with the wake phase. (n = 10–13 for each group, two-way ANOVA, F1, 43 = 4.405, p = 0.0417 with Sidak’s multiple comparison test, WT: Sleep versus WT: Wake, p = 0.02)

(C) Experimental design. Mice (P42–56) were sleep-deprived at the beginning of the light phase for 6 h before recording.

(D) Sleep deprivation (SD) increased tonic inhibition in CA1 neurons in WT mice, and Shisa7 KO abolished SD-induced enhancement of tonic currents (n = 12–22 for each group, two-way ANOVA, F1, 43 = 9.003, p = 0.0039 with Sidak’s multiple comparison test, WT: Ctrl versus WT: SD, p = 0.0001)

(E) Experimental design. In utero electroporation (IUE) to express Shisa7 S405A was performed at E14.5–15.5. Whole-cell recordings in CA1 neurons were performed at P49–P56 after 6-h SD.

(F) Representative image showing mosaic expression of Shisa7 S405A-IRES-GFP in CA1 neurons in an acute hippocampal slice.

(G) Expression of Shisa7 S405A blocked SD-induced potentiation of tonic currents in WT

CA1 neurons (n = 7–11 for each group, one-way ANOVA, F = 6.409, p = 0.0018 with Tukey’s multiple comparisons test, Ctrl versus Ctrl + SD, p = 0.0086).

*p < 0.05 and **p < 0.01. All data are presented as mean ± SEM. See also Figures S5–S7.
neurons, tonic inhibitory currents mediated by $\alpha_4\beta_1$-GABA$_A$Rs are not altered, suggesting a Shisa7-independent pathway for the regulation of trafficking of these extrasynaptic GABA$_A$Rs. Together, these data reveal a key molecular pathway for the regulation of tonic inhibition and underscore the importance of Shisa7-dependent modulation of both synaptic and extrasynaptic GABA$_A$Rs (Han et al., 2019).

Although the molecular mechanisms underlying the regulation of $\alpha_5$-GABA$_A$R trafficking in neurons are not fully understood, recent studies have identified a number of proteins that interact with the $\alpha_5$ subunit to regulate subcellular targeting. For example, it has been shown that radixin, a cytoskeletal protein linking actin to the plasma membrane, interacts with $\alpha_5$-GABA$_A$Rs and regulates $\alpha_5$-GABA$_A$R clustering at the extrasynaptic membrane (Hausrat et al., 2015; Loebrich et al., 2006). However, radixin is not required for $\alpha_5$-GABA$_A$Rs trafficking to the cell surface, and thus, in radixin KO hippocampal neurons, $\alpha_5$-GABA$_A$R clustering is lost, but tonic inhibition is not altered (Hausrat et al., 2015). In addition, gephyrin interaction with $\alpha_5$-GABA$_A$Rs is important for synaptic localization of the receptors (Brady and Jacob, 2015). A recent study has identified a transmembrane protein, cleft lip and palate transmembrane protein 1 (Clptm1), that interacts with both synaptic and extrasynaptic GABA$_A$Rs and negatively regulates inhibitory transmission and tonic inhibition through trapping the receptors in the endoplasmic reticulum (ER) and Golgi apparatus (Ge et al., 2018). However, Clptm1 interaction with extrasynaptic GABA$_A$Rs lacks subunit specificity, binding to $\alpha_4$, $\alpha_5$, and $\delta$ subunits (Ge et al., 2018). It has also been reported that glycine receptors interact with $\alpha_5$-GABA$_A$Rs and regulate $\alpha_5$-GABA$_A$R-mediated currents in both heterologous cells and neurons in the hypoglossal nucleus (Zou et al., 2019). Currently, how Shisa7 functionally interacts with these molecular pathways for the regulation of $\alpha_5$-GABA$_A$R-mediated tonic inhibition remains unknown. Future investigation of $\alpha_5$-GABA$_A$R trafficking in the context of $\alpha_5$-GABA$_A$R native complexes with different binding molecules will provide additional insight into the regulation of tonic inhibition.

A number of studies have shown that protein kinases modulate inhibitory tonic currents in different types of neurons, typically through direct phosphorylation of GABA$_A$R subunits (Connelly et al., 2013; Nakamura et al., 2015). For $\alpha_5$-GABA$_A$R-mediated tonic inhibition, a previous study has shown that CaMKII activity is required for surface expression of $\beta_3|3$-containing GABA$_A$Rs through phosphorylation of $\beta_3$ S383 (Saliba et al., 2012). The regulation of tonic inhibition by PKC has also been well documented, especially for $\alpha_4\delta$-containing GABA$_A$Rs (Abraman et al., 2010, 2014; Bright and Smart, 2013b; Choi et al., 2008). However, the role of PKC in $\alpha_5$-GABA$_A$R-mediated tonic currents is less clear. It has been reported that stimulation of PKC does not change surface expression of $\alpha_5$ in hippocampal neurons (Abraman et al., 2014), although whether inhibition of PKC activity would alter $\alpha_5$-GABA$_A$R-mediated tonic inhibition was unknown. The role of PKA in tonic inhibition mediated by $\alpha_5$-GABA$_A$Rs appears to be cell-type specific in different populations of striatal medium spiny neurons, and the mechanisms underlying the regulation of tonic inhibition by PKA remain largely unclear (Jansen et al., 2009). Our data have now shown that blockade of PKA, PKC, or CaMKII activity leads to a significant reduction of tonic inhibitory currents in WT hippocampal neurons. Intriguingly, Shisa7 KO abolishes the effects of pharmacological inhibition of PKA or PKC, but not CaMKII, activities on tonic inhibition, indicating that Shisa7 mediates the regulatory action of PKA or PKC on tonic inhibition. We have also identified two resides, S405 and S430, in the Shisa7 C terminus that can be phosphorylated by PKA. Significantly, Shisa7 S405 is crucial for $\alpha_5$-GABA$_A$R exocytosis, highlighting a post-translational switch in Shisa7 that is important for regulation of tonic inhibition. Finally, although we have shown the importance of PKC in Shisa7 regulation of tonic inhibition in hippocampal neurons, the mechanisms underlying PKC function remain to be determined.

**Shisa7 and activity-dependent homeostatic plasticity of tonic inhibition**

Chronic pharmacological manipulation of neuronal activity can induce homeostatic adaptations of excitatory and inhibitory transmission, which are powerful mechanisms controlling neuronal excitability and neural network function (Turrigiano, 2012). However, homeostatic plasticity of tonic inhibition is much less investigated. Our data have shown that tonic inhibition in hippocampal neurons exhibits classic homeostatic plasticity. Indeed, a chronic increase or decrease of neuronal activity by BIC or TTX, respectively, triggers activity-dependent bidirectional regulation of $\alpha_5$-GABA$_A$R expression at the neuronal surface and tonic inhibition. Importantly, homeostatic upregulation, but not downregulation, of tonic inhibition requires PKA and Shisa7 S405, indicating that Shisa7 S405-dependent $\alpha_5$-GABA$_A$R exocytosis underlies homeostatic potentiation of tonic inhibition in response to a chronic, global increase of neural activity. In addition, a pharmacological increase of neural activity in vivo significantly enhances tonic inhibition in hippocampal CA1 neurons in WT, but not in Shisa7 KO, mice, showing that Shisa7-dependent homeostatic upregulation of tonic inhibition operates both in vitro and in vivo. Thus, Shisa7 is not only important for maintenance of basal tonic inhibitory currents but also critical for activity-dependent regulation of tonic inhibition in hippocampal neurons. Collectively, our data demonstrate a powerful Shisa7-dependent homeostatic mechanism regulating tonic inhibition that contributes to maintaining a normal range of neuronal excitability in response to chronic changes in neural network activity.

Alternatively, acute changes in neuronal activity within minutes have been shown to regulate $\alpha_5$-GABA$_A$R abundance at the cell surface and tonic inhibition through a pathway involving calcium influx through L-type calcium channels and subsequent CaMKII activation (Saliba et al., 2012). In this scenario, CaMKII activation leads to phosphorylation at $\beta_3$ S383, promoting surface insertion of $\beta_3|3$-containing receptors and enhancing tonic inhibitory currents in hippocampal neurons (Saliba et al., 2012). In agreement, our study has also shown an important role of CaMKII in regulating tonic inhibition. Interestingly, however, our data demonstrate that CaMKII regulation of tonic inhibition operates in a Shisa7-independent manner, whereas homeostatic upregulation of $\alpha_5$ surface expression and tonic inhibition in response to chronic increases in activity require Shisa7, and more specifically, Shisa7 S405, a PKA substrate. Thus, chronic change of neuronal activity triggers a molecular pathway requiring Shisa7 and PKA, whereas acute manipulation of neuronal activity engages a distinct signaling pathway.
pathway dependent on CaMKII and β3 phosphorylation. Together, these data reveal discrete signaling mechanisms in hippocampal neurons regulating tonic inhibition in both an activity-dependent and temporal-specific manner.

**Shisa7 and regulation of tonic inhibition by sleep**

Accumulating evidence has supported the importance of tonic inhibition in regulating a variety of brain functions, including sensory processing (Chadderton et al., 2004; Duguid et al., 2012), motor coordination (Clarkson et al., 2010; Egawa et al., 2012; Woo et al., 2018), and learning and memory (Collinson et al., 2002; Shen et al., 2010; Wang et al., 2012; Wu et al., 2014; Zurek et al., 2014). Dysregulation of tonic inhibition has also been implicated in a number of neurodegenerative and neuropsychiatric disorders (Brickley and Mody, 2012; Glykys and Mody, 2007; Jacob, 2019; Martin et al., 2009; Mohamad and Has, 2019). However, the molecular mechanisms underlying the regulation of tonic inhibition in the context of animal behavior remain largely unknown. On the other hand, sleep is a fundamental physiological process essential for normal brain functioning in animals. It has been proposed that during the daily sleep/wake cycle, homeostatic plasticity of synaptic connections and neuronal excitability occur and play crucial roles in learning and memory (Cirelli, 2017; de Vivo et al., 2017; Diering et al., 2017; Hengen et al., 2016; Tononi and Cirelli, 2014). However, how tonic inhibition is involved in and regulated by the sleep/wake cycle remains unclear. Answers to these questions will not only provide insight into the molecular pathways regulated by sleep but also offer potential specific therapeutic targets for the treatment of sleep disorders.

Our data show that tonic inhibition in hippocampal CA1 neurons is dynamically modulated during the daily sleep/wake cycle. Specifically, tonic inhibition mediated by α5-GABA<sub>A</sub>Rs in wake is significantly higher than that in sleep. Importantly, the increase of tonic inhibition in the wake state is abolished in Shisa7 KO mice, and SD enhances tonic inhibition in WT, but not in Shisa7 KO mice. Thus, the upregulation of tonic inhibition in CA1 neurons associated with the wake state requires Shisa7. Furthermore, Shisa7 S405 is critical for potentiation of tonic inhibition induced by SD, indicating that Shisa7 S405-dependent exocytosis of α5-GABA<sub>A</sub>Rs underlies upregulation of tonic inhibition in CA1 neurons during the wake state.

We noticed that in young adult mice (6–8 weeks old), tonic inhibitory currents in hippocampal CA1 neurons were smaller than that in juvenile mice between 2 and 3 weeks old. A similar change of tonic inhibition during development has previously been reported (Al-Muhtasib et al., 2018; Chudomel et al., 2015; Holter et al., 2010; Pandit et al., 2017). Our data indicate that the decrease of tonic inhibition in hippocampal CA1 neurons in WT young adult mice is due to a significant reduction of the component of α5-GABA<sub>A</sub>R-mediated tonic currents. Therefore, it is plausible that the diminished contribution of α5-GABA<sub>A</sub>Rs to tonic inhibition in hippocampal CA1 neurons in young adult mice might account for the little change of tonic inhibition in Shisa7 KO mice at this developmental stage. Intriguingly, enhancement of tonic inhibition in the wake phase or induced by SD in young adult mice requires Shisa7-medi- ated insertion of α5-GABA<sub>A</sub>Rs, suggesting that although Shisa7 is not necessary for the basal tonic inhibition, it is critical for activity-dependent α5-GABA<sub>A</sub>R trafficking and upregulation of tonic inhibition. Future experiments examining the mechanisms underlying Shisa7 independent regulation of α5-GABA<sub>A</sub>R-mediated basal tonic inhibition in young adult mice will provide additional insight into dynamic regulation of tonic inhibition in vivo.

A recent study has reported that inhibitory transmission changes over the daily sleep/wake cycle. Specifically, in hippocampal CA1 and cortical pyramidal neurons synaptic inhibition is increased, whereas synaptic excitation is decreased, leading to altered excitation/inhibition balance during the sleep phase (Bridi et al., 2019). Thus, it appears that different molecular and cellular mechanisms exist in neurons governing the regulation of inhibitory synaptic transmission and tonic inhibition over the daily sleep/wake cycle. Currently, the functional significance of increase of tonic inhibition in wake remains to be determined. It has been reported that tonic inhibition helps set the threshold for the induction of long-term potentiation at excitatory synapses (Martin et al., 2010) and thus may enhance selectivity during learning in wake (Tononi and Cirelli, 2014). Interestingly, tonic inhibition has also been shown to be dynamically modulated by a variety of physiological and pathological processes in vivo, including learning, puberty, the ovarian cycle, pregnancy, epilepsy, and acute stress (Cushman et al., 2014; Maguire and Mody, 2008; Maguire et al., 2005; Peng et al., 2004; Serra et al., 2006; Shen et al., 2007, 2010; Zhang et al., 2007), indicating that plasticity of tonic inhibition is broadly implicated in animal behavior and cognition. However, the role of Shisa7 in the dynamic regulation of tonic inhibition triggered by these behavioral and cognitive processes remains to be determined.

In summary, we have uncovered a critical role of Shisa7 in the regulation of α5-GABA<sub>A</sub>R exocytosis and tonic inhibition in juvenile mice, identified a molecular pathway controlling activity-dependent modulation of tonic inhibitory currents, and revealed a link between the daily sleep/wake cycle and tonic inhibition regulation in young adult mice. As dysfunctions in tonic inhibition have been implicated in a number of neurological and psychiatric disorders and extrasynaptic GABA<sub>A</sub>Rs are important drug targets for the treatment of epilepsy, depression, and cognitive impairment (Brickley and Mody, 2012; Glykys and Mody, 2007; Jacob, 2019; Martin et al., 2009; Mohamad and Has, 2019), our findings also provide insight to design therapeutic reagents for intervening and treating brain disorders.

**Limitations of study**

Finally, caution should be taken in the interpretation of the regulation of tonic inhibition by the daily sleep/wake cycle. Although we have defined sleep and wake using a PiezoSleep behavioral tracking system, our approach to measure sleep patterns is indirect and may not fully capture the real-time sleep pattern and duration. In addition, different criteria and approaches have been employed to measure sleep in rodents in the literature (Bridi et al., 2019; de Vivo et al., 2017; Diering et al., 2017; Liu et al., 2010), indicating a need in establishing a well-validated, noninvasive system to rapidly analyze sleep for subsequent electrophysiological or biochemical investigations in the future. Furthermore, we could not completely rule out the potential influence on tonic
inhibitory currents by circumstantial factors associated with the in vitro preparation of mouse brain slices. Considering these, while our data show a significant change of tonic currents over the daily sleep/wake cycle, future work toward a more complete understanding of sleep-dependent regulation of tonic inhibition will be necessary and invaluable.

**STAR METHODS**

Detailed methods are provided in the online version of this paper and include the following:

- **KEY RESOURCES TABLE**
- **RESOURCE AVAILABILITY**
  - Lead contact
  - Materials availability
  - Data and code availability
- **EXPERIMENTAL MODEL AND SUBJECT DETAILS**
  - Animals
  - Cell lines
  - Dissociated hippocampal neuronal culture
- **METHOD DETAILS**
  - Plasmids
  - Cell transfection
  - Immunocytochemistry
  - Image acquisition
  - Live imaging with FRAP-FLIP
  - Co-immunoprecipitation and western blot
  - GST fusion protein production and in vitro phosphorylation
  - Mass spectrometry analysis
  - Electrophysiology
  - In utero electroporation
  - Piezoelectric sleep recording
  - Sleep deprivation
- **QUANTIFICATION AND STATISTICAL ANALYSIS**
  - Image analysis
  - Statistical analysis

**SUPPLEMENTAL INFORMATION**

Supplemental information can be found online at https://doi.org/10.1016/j.celrep.2021.108899.

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**AUTHOR CONTRIBUTIONS**

K.W. and W.L. designed the project, and W.L. supervised the project. K.W. performed imaging, biochemical, electrophysiological, and behavioral experiments. K.W. and Y.L. performed MS assays. W.H. performed some of electrophysiological assays in HEK293T cells. Q.T performed neuronal cultures and in utero electroporation (IUE). W.L. and K.W. wrote the manuscript, and all authors read and commented on the manuscript.

**DECLARATION OF INTERESTS**

The authors declare no competing interests.

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### STAR★METHODS

#### KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| **Antibodies**      |        |            |
| Rabbit Polyclonal Anti-Shisa7 | GenScript | Han et al., 2019 |
| Mouse Monoclonal Anti-Flag M2, Clone M2 | Sigma-Aldrich | Cat# F3165; RRID: AB_269529 |
| Rabbit Polyclonal Anti-GFP | Sigma-Aldrich | Cat# G1544; RRID: AB_439690 |
| Mouse Monoclonal Anti-GABA(A) α5 Receptor | James Trimmer, University of California at Davis | Cat# N415/24; RRID: AB_2750794 |
| Rabbit Polyclonal Anti-GABA(A) δ Receptor | Alomone Labs | Cat# AGA-014; RRID: AB_2340938 |
| Rabbit Polyclonal Anti-GABA(A) γ4 Receptor | Alomone Labs | Cat# AGA-008; RRID: AB_10917596 |
| Rabbit Polyclonal Anti-GABA(A) δ5 Receptor | Synaptic Systems | Cat# 224503; RRID: AB_2619944 |
| Rabbit IgG | Sigma-Aldrich | Cat# I8140; RRID: AB_1163661 |
| Mouse Monoclonal Anti-Gephyrin | Synaptic Systems | Cat# 147011; RRID: AB_887717 |
| Chicken Polyclonal Anti-MAP2 | Aves Labs | Cat# MAP; RRID: AB_2313549 |
| Alexa 647 goat anti-chicken IgG (H+L) | Thermo Fisher Scientific | Cat# A-21449; RRID: AB_2535866 |
| Alexa 488 goat anti-rabbit IgG (H+L) | Jackson ImmunoResearch Labs | Cat# 111-546-003; RRID: AB_2338053 |
| Alexa 488 goat anti-mouse IgG (H+L) | Jackson ImmunoResearch Labs | Cat# 115-546-003; RRID: AB_2338859 |
| Alexa 555 donkey anti-rabbit IgG (H+L) | Thermo Fisher Scientific | Cat# A-31572; RRID: AB_162543 |
| **Chemicals, peptides, and recombinant proteins** | | |
| NeuroMag reagent | Oz Biosciences | Cat# NM51000 |
| CalPho Mammalian Transfection Kit | Takara | Cat# 631312 |
| Lipofectamine 3000 | Thermo Fisher Scientific | Cat# L3000008 |
| Bicuculline | Abcam | Cat# ab120110 |
| L655,708 | Sigma-Aldrich | Cat# L9787 |
| 4,5,6,7-tetrahydroisoxazolo(5,4-c) pyridin-3-ol (THIP) | Santa Cruz | Cat# SC204342 |
| H89 | Abcam | Cat# ab120341 |
| KN62 | Abcam | Cat# ab120421 |
| GF109203X | Abcam | Cat# ab144264 |
| D-APV | Abcam | Cat# ab120003 |
| DNXQ | Alomone labs | Cat# D-131 |
| Tetrodotoxin (TTX) | Alomone Labs | Cat# T-590 |
| Anti-Flag M2 affinity gel | Sigma-Aldrich | Cat# A2220; RRID: AB_10063035 |
| Phosphatase Inhibitor Cocktail | Thermo Fisher Scientific | Cat# 78420 |
| Protease inhibitor cocktail | Roche | Cat# 05892791001 |
| Kainic acid | Abcam | Cat# ab120100 |
| Pentyleneetetrazole (PTZ) | Sigma-Aldrich | Cat# P6500 |
| Pierce Glutathione Agarose | Thermo Fisher Scientific | Cat# 16101 |
| Dynabead Protein G | Thermo Fisher Scientific | Cat# 10003D |
| cAMP-Dependent Protein Kinase, Catalytic Subunit | Promega | Cat# V5161 |
| Protein Kinase G | Promega | Cat# V5261 |
| **Experimental models: cell lines** | | |
| Primary cultures of hippocampal neurons | This paper | N/A |
| HEK293T | ATCC | Cat# CRL-1126 |

(Continued on next page)
RESOURCE AVAILABILITY

Lead contact
Further information and requests for resources and reagents should be directed to the Lead Contact, Wei Lu (luw4@mail.nih.gov).

Materials availability
All unique reagents generated in this study are available from the Lead Contact with a completed Materials Transfer Agreement.

Data and code availability
This study did not generate datasets/code.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Animals
All animal handling was performed in accordance with animal protocols approved by the Institutional Animal Care and Use Committee (IACUC) at NIH/NINDS. C57BL/6 mice were purchased from Charles River and Shisa7 germline knockout (KO) mice were generated as described previously (Han et al., 2019). All mice were housed and bred in a conventional vivarium with ad libitum access to food and water under a 12-h circadian cycle. Time-pregnant mice at E17.5-18.5 were used for dissociated hippocampal culture. Time-pregnant mice at E14.5-15.5 were used for in utero electroporation (IUE). Mice of both sexes at P16-21 were used for biochemical experiments. To investigate the effects of Shisa7 KO on tonic inhibition in vivo, mice of both sexes at P16-21 were used to prepare acute hippocampal slices for electrophysiology experiments. To evaluate the effects of sleep/wake cycle or sleep deprivation (SD) on tonic inhibition, young adult male mice (6-8 weeks old) were used for electrophysiology experiments.
Balanced Salt Solution (HBSS, GIBCO) containing 20 U/ml papain (Worthington) and 100 U/ml DNase I (Worthington) at 37°C for 45 min. After centrifugation for 5 min at 800 rpm, the pellet was resuspended in HBSS containing 100 U/ml DNase I, and was fully dissociated by pipetting up and down. Cells were then transferred into HBSS containing trypsin inhibitor (10 mg/ml, Sigma-Aldrich) and BSA (10 mg/ml, Sigma-Aldrich). After centrifugation for 10 min at 800 rpm, cells were resuspended in Neurobasal media (GIBCO) supplemented with 2% B27 (GIBCO) and 2 mM GlutaMAX (GIBCO) and were plated on poly-D-lysine (Sigma-Aldrich)-coated glass coverslips residing in 24-well plates at a density of 1.5 x 10⁵ cells/well for electrophysiology experiments or 0.8 x 10⁵ cells/well for imaging experiments. Cultures were maintained in Neurobasal media supplemented with 2% B27 and 2 mM GlutaMAX in a humidified incubator at 37°C for 5% CO₂. Culture media were changed by half volume once a week.

**METHOD DETAILS**

**Plasmids**

pCAGGs-Shisa7-IRES-GFP and pcDNA3-Flag-Shisa7 were used as previously described (Han et al., 2019). ß5-GFP was purchased from Addgene (plasmid # 118956). Human GABAR ß5, ß3 and ß2 in pcDNA3.1 Zeo were gifts from Joseph Lynch’s lab at University of Queensland, Australia. The coding sequence of Shisa7 phosphomimic (S405D), phosphodeficient (S306A, S405A, S430A), and ßC-tail mutants were generated separately by overlapping PCR using pcDNA3-Flag-Shisa7 as a template and were then inserted into the pCAGGs-IRES-GFP or pCAGGs-IRES-mCherry vectors. The coding sequence of Superecliptic pHluorin (SEP)-tagged ß5 was obtained by PCR with Human GABAR ß5 as the template and then replaced ß1 coding sequence in SEP-ß1 (Addgene plasmid # 49168). pGEX-4T-Shisa7 (211-349) and pGEX-4T-ßisa7 (347-558) were generated by GenScript. All plasmids were confirmed by DNA sequencing.

**Cell transfection**

HEK293T cells were transfected with ß5ß3ß2 receptors, together with GFP or Shisa7/GFP, using CalPhos Mammalian Transfection Kit (Takara). Electrophysiological recordings or immunostaining were performed 24-48 h after transfection. Hippocampal neurons at DIV13-15 were transfected with SEP-ß5 using Lipofectamine 3000 (Thermo Fisher Scientific) and live imaging was performed 24 h after transfection. Hippocampal neurons at DIV16 were transfected with Shisa7 phosphomimic (S405D), phosphodeficient (S306A, S405A, S430A) or ßC-tail mutants for overexpression and rescue experiments using NeuroMag reagent (Oz Biosciences). Electrophysiological recordings or immunostaining were performed 36-72 h after transfection. All transfection kits were used according to the manufacturer’s instructions.

**Immunocytochemistry**

Transfected HEK293T cells and hippocampal neurons grown on glass coverslips were fixed with 4% paraformaldehyde and 4% sucrose in PBS. For surface ß5 staining, cells were blocked with 10% NGS, washed, and incubated with rabbit ß5 antibody (1:500, Synaptic Systems) in 3% NGS overnight at 4°C, and then washed, and incubated with Alexa 555-conjugated anti-rabbit secondary antibody (1:1000, Thermo Fisher Scientific) in 3% NGS for 1 h. After surface staining, cells were permeabilized with 0.25% Triton X-100 for 15 min, washed, blocked in 10% NGS, and then incubated with mouse ß5 antibody (1:500, James Trimmer, University of California at Davis), mouse gephyrin antibody (1:500, Synaptic Systems) or chicken MAP2 antibody (1:1000, Aves Labs) in 3% NGS overnight at 4°C. Cells were then washed, and incubated with Alexa 488-conjugated anti-mouse secondary antibody (1:1000, Jackson ImmunoResearch Labs) or Alexa 647-conjugated anti-chicken secondary antibody (1:1000, Thermo Fisher Scientific) in 3% NGS for 1 h. Coverslips were then washed for three times with PBS and mounted with Fluoromount-G (Southern Biotech). For the endocytosis assay, hippocampal neurons at DIV16 were incubated live with rabbit ß5 antibody (1:500, Synaptic Systems) at 37°C for 10 min in conditioned culture medium. After incubation, the neurons were washed with PBS and then incubated in antibody-free medium to allow antibody-bound receptors to undergo internalization at 37°C for 30 min, followed by fixation with 4% paraformaldehyde and 4% sucrose in PBS. After fixation, neurons were washed and then blocked with 10% NGS for 1 h, exposed to Alexa 555-conjugated anti-rabbit secondary antibody (1:200, Thermo Fisher Scientific) for 1 h under the nonpermeabilized condition, and then internalized ß5 was labeled with Alexa 488-conjugated anti-rabbit secondary antibody (1:1000, Jackson ImmunoResearch Labs) for 1 h after permeabilization in PBS containing 0.25% Triton X-100 and blocking in 10% NGS. Coverslips were washed for three times with PBS and mounted with Fluoromount-G.

**Image acquisition**

For Immunocytochemistry in fixed neurons, fluorescence images were acquired on a Zeiss LSM 880 laser scanning confocal microscope with a 63 x 1.4 NA oil immersion objective. Multiple z sections (4-5 optical slices) were collected with step intervals of 0.39 μm.
in the z direction. Scan speed function were set to 9 and the mean of four lines was collected. Images were captured using a 1024 × 1024 pixel screen for HEK293T cells and a 1024 × 256 pixel screen for neuronal dendrites. All the parameters used in confocal microscopy were consistent in each experiment, including the laser excitation power, detector, off-set gains, and the pinhole diameter.

**Live imaging with FRAP-FLIP**

For live imaging of α5 receptor exocytosis, fluorescence recovery after photobleaching (FRAP) and fluorescence loss in photobleaching (FLIP) were applied as previously described with minor modifications (Hildick et al., 2012; Nakahata et al., 2017). Briefly, hippocampal neurons at DIV13-15 were transfected with SEP-α5 (and mCherry, Shisa7/mCherry or Shisa7 S405A/mCherry) constructs for 24 h prior to experiments. Fluorescence of SEP-α5 was photobleached using a 488 nm laser (200 mW) at 80% power in a rectangular region of dendrites expressing SEP-α5. Then, repetitive photobleaching (10% laser power) at the edge of the initial photobleaching region was applied throughout the imaging period panel in order to avoid the lateral diffusion of nonbleached surface receptors. FRAP of SEP-α5 in the central region was used to measure receptor exocytosis. Recovery from photobleaching in the central region was monitored by consecutive acquisitions every 30 s for 7 min and normalized to the fluorescence measured before photobleaching.

**Co-immunoprecipitation and western blot**

Transfected HEK293T cells were homogenized in ice-cold lysis buffer containing 25 mM Tris pH 7.5, 1% Triton X-100, 150 mM NaCl, 5% glycerol, 1 mM EDTA and protease inhibitor cocktail (Roche). After incubation on ice for 30 min, the homogenates were centrifuged at 12,000 × g at 4°C for 15 min. The supernatants were collected, and the total protein concentrations were measured using BCA Protein Assay Kit (Thermo Fisher Scientific). For immunoprecipitation, the supernatants were incubated with 40 μL Anti-FLAG M2 Affinity gel (Sigma-Aldrich) at 4°C overnight. The beads were collected by centrifugation and washed three times with lysis buffer. The precipitated proteins were eluted with SDS loading buffer with β-mercaptoethanol, and denatured at 37°C for 10 min before SDS-PAGE and immunoblotting.

For western blot, the proteins were resolved by SDS-PAGE and then transferred onto PVDF membranes. The membranes were blocked, incubated with primary antibodies at 4°C overnight, washed and incubated with HRP-conjugated secondary antibodies for 1 h at room temperature (~23°C). Protein was detected with the standard enhanced chemiluminescence (ECL) method, and documented by a gel imaging system (Li-COR Odyssey).

**GST fusion protein production and in vitro phosphorylation**

The Shisa7 C-terminal domains (amino acids 211-349 and 347-558, respectively) were cloned into pGEX-4T. GST fusion proteins were produced in the BL21 E. coli strain (Thermo Fisher Scientific) by inducing protein production with 50 μM isopropyl β-D-thio-galactopyranoside (IPTG) at 16°C for 10-12 h. E. coli were then lysed with a sonicator in a Tris-buffered saline (TBS) buffer containing protease inhibitors (Roche), 100 μg/ml lysozyme, 1 mM dithiothreitol (DTT) and 0.2 mM EDTA. The fusion proteins were purified using Pierce Glutathione Agarose (Thermo Fisher Scientific) according to manufacturer’s instructions. For PKA in vitro phosphorylation, the fusion proteins were phosphorylated in PKA kinase buffer (10 mM HEPES, 20 mM MgCl₂, and 50 μM ATP) with 50 ng of purified PKA catalytic subunit (Promega). For PKC in vitro phosphorylation, reactions were performed in PKC kinase buffer (20 mM HEPES, pH 7.4, 1.67 mM CaCl₂, 1 mM DTT, 10 mM MgCl₂, and 50 μM ATP) with 10 ng of purified PKC (Promega). All in vitro kinase assays were performed at 30°C for 30 min. The reactions were halted with addition of SDS loading buffer with β-mercaptoethanol and incubation at 65°C for 5 min. The proteins were resolved by SDS-PAGE and then stained with Coomassie blue. Coomassie staining gel were cut out and were analyzed by Mass Spectrometry.

**Mass spectrometry analysis**

Protein samples were reduced with 5 mM TCEP, followed by 5 mM NEM treatment, and digested with trypsin. Digests were used for LC-MS/MS data acquisition on an Orbitrap Lumos mass spectrometer (Thermo Fisher Scientific) coupled with a 3000 Ultimate HPLC instrument (Thermo Fisher Scientific). Peptides were separated using an ES803 column (Thermo Fisher Scientific) with the percentage of mobile phase B (MPB, which contains 98% ACN, 19.9% H₂O, 0.1% FA) increased from 2% to 24% in 38 or 64 min. The LC-MS/MS data were acquired in data-dependent mode with a decision tree method. The MS resolution is 120K at m/z 400, MS scan range is 300-1500 m/z, the automated gain control (AGC) target is 2 × 10⁶. The quadrupole isolation window is 1.6 m/z. Precursors with charge states 2-6 and intensity higher than 1 × 10⁴ within a 3-s cycle between MS1 scans were selected for MS/MS acquisition in the linear ion trap. Mascot database search was performed for PTM analysis. The following parameters were used for samples digested with
For GABA-evoked whole-cell currents, HEK293T cells were co-transfected with z5b3/2 receptors, together with either GFP or Shisa7/GFP plasmids. All recordings were performed after 24-48 h transfection. Coverslips containing HEK293T cells were perfused continuously with an external solution (in mM): 140 NaCl, 5 KCl, 2 CaCl2, 1 MgCl2, 10 HEPES and 10 D-glucose. The internal solution contained (in mM): 70 CsMeSO4, 70 CsCl, 8 NaCl, 10 HEPES, 0.3 Na-GTP, 4 Mg-ATP and 0.3 EGTA (pH 7.3; osmolality 285-290 mOsm). Experiments were started 3–5 min after achieving the whole-cell configuration at −70 mV. Rapid application/removal of saturating GABA (10 mM) was performed using a computer-controlled multi-barrel perfusion system (Automate Scientific). For recording in dissociated hippocampal cultures, neurons growing on coverslips were transferred to a submersion chamber, perfused with artificial cerebrospinal fluid (ACSF) containing (in mM): 130 NaCl, 3.5 KCl, 24 NaHCO3, 1.25 NaH2PO4·H2O, 10 glucose, 2.5 CaCl2 and 1.5 MgCl2 supplemented with 0.5 μM TTX (Alomone Labs), 20 μM DNOX (Alomone labs) and 50 μM D-APV (Abcam) without exogenous GABA. The intracellular solution contained (in mM): 70 CsMeSO4, 70 CsCl, 8 NaCl, 10 HEPES, 0.3 Na-GTP, 4 Mg-ATP, and 0.3 EGTA (pH 7.3; osmolality 285-290 mOsm). In some recordings as indicated, HB9 (20 μM, Abcam), GF 109203X (200 nM, Abcam) or KN62 (3 μM, Abcam) was added directly into intracellular solution to inhibit PKA, PKC or CaMKII, respectively via intra-pipette administration. To induce homeostatic plasticity in vitro, neurons were treated with 1 μM TTX or 40 μM bicuculline for 48 h prior to electrophysiological recording. For recording in acute brain slices, transverse hippocampal slices (300 μm thickness) were prepared from 16-21 days old mice of both sexes or 6-8 weeks old male mice in chilled high sucrose cutting solution, containing (in mM): 130 CsCl, 8.5 NaCl, 5 HEPES, 4 MgCl2, 4 Na-ATP, 0.3 Na-GTP and 1 QX-314 (pH 7.3; osmolality 285-290 mOsm). To induce activity elevation in vivo, mice at P16 were intraperitoneally injected with kainic acid (KA, 4 mg/kg), pentylentetrazol (PTZ, 40 mg/kg) or as control, an equal volume of saline. Hippocampal CA1 pyramidal cells were recorded 6 h after KA injection or 24 h after PTZ injection. The experimenters were not blinded to the genotype or the treatment groups.

To measure tonic inhibitory currents in neuronal cultures or in acute hippocampal slices, the GABAR competitive antagonist bicuculline (20 μM, Abcam) was bath applied after obtaining a stable baseline recording at a holding potential of −70 mV. Custom-written macros running under Igor Pro (WaveMetrics) were used to determine the values of tonic currents. An all-points histogram was plotted for a 20-s period before and during bath-application of bicuculline, fitting the histogram with a Gaussian distribution gave the mean baseline holding currents, and the difference in baseline holding currents before and during bicuculline application was calculated to be the tonic currents. In some recordings as indicated, L-655,708 (100 nM, Sigma-Aldrich) or THIP (10 μM, Santa Cruz) was added to the ACSF via perfusion and their effects on tonic currents were recorded. Tonic currents were normalized to membrane capacitance, to account for variability in cell size. Membrane capacitance was obtained using the voltage step method as described previously (Gentet et al., 2000). Series resistance was monitored and not compensated, and cells in which series resistance was more than 25 MΩ or varied by 25% during a recording session were discarded. Whole-cell recordings were obtained from cells visualized with a fixed stage upright microscope (BX51WI, Olympus). Fluorescence-positive cells were identified by epifluorescence microscopy. All recordings were performed at room temperature. Data were collected with a Multiclamp 700B amplifier (Axon Instruments), filtered at 2 kHz, and digitized at 10 kHz.

In utero electroporation

In utero electroporation was performed as described previously (Li et al., 2017). Briefly, E14.5-15.5 timed-pregnant mice were anesthetized and their uterine horns were exposed with a midline laparotomy incision. Embryos were gently pulled outside the abdominal cavity. A volume of 2 μL of expression constructs Shisa7 S405A/GFP (2 μg/μL) plus 0.05% fast green (Sigma-Aldrich) was injected into the lateral ventricles of the embryonic brain with a glass micropipette. For electroporation, 5 × 50 ms, 45 V square pulses separated by
950 ms intervals were delivered with forceps-type electrodes connected to an ECM 830 electroporator (BTX Harvard Apparatus). The uterus was then returned to the abdominal cavity, and Buprenex (0.1 mg/kg) was applied before the wound was sutured. The pregnant mouse was warmed in an incubator until it became conscious. Ketoprofen (5 mg/kg) was administered daily for two days after surgery.

**Piezoelectric sleep recording**

Sleep-wake activity was recorded using a piezoelectric monitoring system (Signal Solutions) as described with minor modifications (Holth et al., 2019; Hou et al., 2019; Mang et al., 2014). It has been demonstrated that this sensitive system estimated total sleep time with more than 90% accuracy compared to EEG, although it cannot distinguish rapid eye movement (REM) sleep from non-rapid eye movement (NREM) (Mang et al., 2014). Prior to piezoelectric recording, 6-8 weeks old male mice were singly housed and habituated to the recording cage with free access to food and water for 2 days under a 12-h circadian cycle. During piezoelectric recording, mice were left undisturbed and the piezo-electric signals in 2-s epochs were automatically analyzed by a linear discriminant classifier algorithm and classified as sleep or wake, as detailed in previous studies (Hou et al., 2019; Mang et al., 2014). Total sleep percentages and hourly sleep percentages were calculated using SleepStats Data Explorer (Signal Solutions). Based on the sleep pattern we recorded (Figure S5) and the criteria used in the previous study [73], we defined sleep/wake mice as follows: “sleep mice” were asleep for at least 65% of the previous 4 h (≥60% per hour), whereas “wake mice” were awake for at least 75% of the previous 4 h (≥70% per hour). Mice were selected for electrophysiology experiments only if they met the criteria. For the mice used in all other experiments not related to the sleep/wake behavioral experiments, we didn’t measure their sleep-wake behavior before slicing, and mice were pooled for following experiments.

**Sleep deprivation**

Mice were sleep-deprived for 6 h by gentle handling starting at the light onset. At the beginning of the sleep deprivation period, mice were taken from their home cages and transferred to a new cage individually and were gently handled (by gentle tapping of the cage, disturbing the bedding materials or gentle prodding mouse with paintbrush) every time they were falling asleep. Sleep deprivation was continued until the end of the sixth hour. As a control, mice were undisturbed during the first 6 h in the light phase. After sleep deprivation, mice were sacrificed and subjected to electrophysiological recording.

**QUANTIFICATION AND STATISTICAL ANALYSIS**

**Image analysis**

For fluorescence intensity analysis, maximal projection images were created with LSM880 browser software (Zeiss) from 5-6 serial optical sections. Image analysis was performed using ImageJ (NIH). The fluorescence intensity was determined from fluorescent signal above a threshold set for distinguishing cell morphology from background. For quantification of fluorescence intensity, region-of-interest (ROI) was defined along a segment of the dendrite (30-35 μm), or an entire HEK293T cell. Background intensity was subtracted by measuring a cell-lacking region in each image. The average values of fluorescence intensities in ROI (the total fluorescence intensity divided by the total area of a dendritic segment or a HEK293T cell) were calculated by ImageJ. The average fluorescence intensity in each group was normalized to their control group.

**Statistical analysis**

For all biochemical, cell biological and electrophysiological recordings, at least three independent experiments were performed (independent cultures, transfections or different mice). Statistical analysis was performed in GraphPad Prism 8.0 software. Normality distribution was tested by the Shapiro-Wilk test before carrying out a subsequent statistical test. Direct comparisons between two groups were made using two-tailed Student’s t test or Mann-Whitney U test. Multiple comparisons were performed using one-way ANOVA, Kruskal-Wallis test or two-way ANOVA with corrections for multiple comparisons test (see figure legends for specifics). For power analysis, G’Power was used to analyze the sample size. The statistical significance was defined as *p < 0.05, **p < 0.01, ***p < 0.001 or ****p < 0.0001, respectively. All data are presented as mean ± SEM.
Supplemental information

Activity- and sleep-dependent regulation of tonic inhibition by Shisa7

Kunwei Wu, Wenyan Han, Qingjun Tian, Yan Li, and Wei Lu
Figure S1. Tonic currents and capacitance in WT and Shisa7 KO hippocampal neurons, Related to Figure 1

Genetic deletion of Shisa7 significantly reduced tonic currents in cultured hippocampal neurons (A), in CA1 hippocampal neurons (B), and in DG granule cells (C). There was no significant difference in the capacitance between WT and Shisa7 KO neurons. (n = 15-19 for each group, cultured hippocampal neurons: t test, p = 0.0086; CA1 hippocampal neurons: t test, p < 0.0001; DG granule cells: t test, p = 0.0012)

**p < 0.01, ****p < 0.0001. All data were presented as mean ± SEM.
Figure S2. Co-immunoprecipitation, endocytosis and exocytosis assays, Related to Figure 2
(A) Bar graph showing the quantitation of the data in Figure 2A (n = 3, one-way ANOVA, F = 14.58, p < 0.005 with Tukey's multiple comparisons test. p = 0.0087).

(B) Bar graph showing the quantitation of the data in Figure 2B (n = 3, one-way ANOVA, F = 216.6, p < 0.001 with Tukey's multiple comparisons test. p < 0.0001).

(C) Immunostaining (left) showing the distribution of α5 and GABAergic postsynaptic marker gephyrin in cultured hippocampal neurons. Summary graph (right) showing percentages of α5 clusters that were colocalized with gephyrin (Gephyrin⁺ α5⁺, ~14%) and were not colocalized with gephyrin (Gephyrin⁻ α5⁻, ~86%) (n = 24).

(D) Endocytosis assay of α5-GABAₐRs in hippocampal cultures. Surface α5-GABAₐRs (Sα5) are labeled in red, and internalized α5-GABAₐRs (Iα5) are in green. Bar graphs in the right showing that α5 endocytosis was indistinguishable between WT and Shisa7 KO hippocampal neurons (WT, n = 19; KO, n = 17).

(E) A representative image of a neuron transfected with SEP-α5 (left). A portion of dendritic branches (yellow box) was used for live imaging of SEP-α5 exocytosis. A schematic of hypothetical SEP-α5 movements under the FRAP-FLIP experiments (right). A portion of a dendritic branch was bleached (yellow box) in order to remove surface SEP-α5 fluorescence. SEP-α5 fluorescence was measured in the center of bleached region (red box) for 7 min afterwards. During this period, repeated bleaching (FLIP) was applied to the bilateral regions (white box) to prevent lateral diffusion of non-bleached receptors into the center area (red box). **p < 0.01, ****p < 0.0001. All data were presented as mean ± SEM.
Figure S3. S405 is required for Shisa7-induced increase of tonic inhibition, Related to Figure 4

(A) Experimental design. Cultured hippocampal neurons were transfected at DIV16 for 36-72 h and then were recorded for tonic currents.

(B) Overexpression of Shisa7 or Shisa7 S405D, but not Shisa7 ΔC or Shisa7 S405A, increased tonic currents in WT hippocampal neurons. (n = 9-12 for each group, one-way ANOVA, F = 17.65, p < 0.0001 with Dunnett's multiple comparisons test. GFP versus Shisa7/GFP, p = 0.0174; GFP versus Shisa7ΔC/GFP, p = 0.006; GFP vs. Shisa7 S405A/GFP, p = 0.0091; GFP versus Shisa7 S405D/GFP, p = 0.0163)

*p < 0.05, **p < 0.01. All data were presented as mean ± SEM.
Figure S4. Bicuculline treatment increases \( \alpha_5 \)-GABA\(_A\)R-mediated tonic currents, related to Figure 5

(A) Representative traces showing tonic currents in cultured hippocampal neurons with or without BIC treatment for 48 h. Before application of bicuculline to block all GABA\(_A\)Rs, L655,708 (100 nM) was applied to block \( \alpha_5 \)-GABA\(_A\)R during recording.

(B) BIC treatment increased L655,708-sensitive, but not L655,708-insensitive, tonic currents. (Ctrl, \( n = 10 \); BIC, \( n = 11 \), t test, \( p = 0.0017 \)).

**\( p < 0.01 \). All data were presented as mean ± SEM.
Figure S5. Utilization of a PiezoSleep mouse behavioral tracking system to monitor sleep and wake patterns in WT and Shisa7 KO mice, Related to Figure 7

Sleep and wake states were determined by a PiezoSleep mouse behavioral tracking system. Percentage of sleep time were plotted hourly over 24 h in WT and Shisa7 KO mice. Percentage of sleep time spent in the whole day (Total) and in the dark phase (Dark) was shortened in Shisa7 KO mice, compared to WT mice. (WT, n = 11; KO, n = 12, Total: t test, p = 0.0008; Dark: Mann-Whitney U test, p = 0.026). We defined sleep/wake mice as follows: “sleep mice” were asleep for at least 65% of the previous 4 h (≥60% per hour), whereas “wake mice” were awake for at least 75% of the previous 4 h (≥70% per hour). Mice were selected for electrophysiological experiments only if they met the criteria. Above the dotted line in red showed the time points when the mice were asleep at least 60% per hour and below the dotted line in green showed the time points when the mice were awake at least 70% per hour.

*p < 0.05, ***p < 0.001. All data were presented as mean ± SEM.
Figure S6. Shisa7 is required for sleep deprivation-induced increase of α5-GABA$_A$R-mediated tonic currents in young adult mice, Related to Figure 7

(A) Representative traces showing tonic currents in hippocampal CA1 neurons of control or sleep-deprived mice. Before application of bicuculline to block all GABA$_A$Rs, L655,708 (100 nM) was applied to block α5-mediated tonic currents during recording.

(B) SD increased L655,708-sensitive, but not L655,708-insensitive, tonic currents in WT neurons. Genetic deletion of Shisa7 abolished the effect of SD on L655,708-sensitive tonic currents. (n = 6-20 for each group. two-way ANOVA, F$_{1,40}$ = 4.909, p = 0.03 with Sidak's multiple comparison test, WT: Ctrl versus WT: SD, p = 0.0011)

(C) Bar graph showing tonic currents in WT young adult mice (6-8 weeks old) were smaller than those recorded in P16-21 juvenile mice. (n = 15-22 for each group. Mann-Whitney U test, p = 0.0017)

(D) Bar graph showing L655,708-sensitive, but not L655,708-insensitive, tonic currents in WT CA1 pyramidal neurons was reduced in young adult mice (6-8 weeks old), as compared with those in P16-21 juvenile mice. (n = 11-20 for each group. Mann-Whitney U test, p < 0.0001)

(E) Bar graph showing L655,708-sensitive and -insensitive, tonic currents in P16-21 juvenile mice and young adult mice. (n = 11-20 for each group)

(F) Bar graph showing the proportion of L-655,708-sensitive tonic currents was significantly decreased in young adult mice, as compared with that in P16-21 juvenile mice. (n = 11-20 for each group, t test, p < 0.0001).

**p < 0.01, ****p < 0.0001. All data were presented as mean ± SEM.
Figure S7. Sleep deprivation increases phosphorylation of Shisa7 at S405, Related to Figure 7

Quantification of abundance of Shisa7 phospho S405 in hippocampal lysates prepared from control and SD mice by mass spectrometry (Ctrl, n = 5; SD, n = 4, t test, p = 0.0338).

*p < 0.05. All data were presented as mean ± SEM.