Wip1 phosphatase modulates both long-term potentiation and long-term depression through the dephosphorylation of CaMKII

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ABSTRACT
Synaptic plasticity is an important mechanism thatunderlies learning and cognition. Protein phosphorylation by kinases and dephosphorylation by phosphatases play critical roles in the activity-dependent alteration of synaptic plasticity. In this study, we report that Wip1, a protein phosphatase, is essential for long-term potentiation (LTP) and long-term depression (LTD) processes. Wip1-deficiency-induced aberrant elevation of CaMKII T286/287 and T305 phosphorylation underlies these dysfunctions. Moreover, we showed that Wip1 modulates CaMKII dephosphorylation. Wip1/−/− mice exhibit abnormal GluR1 membrane expression, which could be reversed by the application of a CaMKII inhibitor, indicating that Wip1/CaMKII signaling is crucial for synaptic plasticity. Together, our results demonstrate that Wip1 phosphatase plays a vital role in regulating hippocampal synaptic plasticity by modulating the phosphorylation of CaMKII.

KEYWORDS
CaMKII; GluR1; hippocampus; LTD; LTP; Wip1

Introduction
Synaptic plasticity is an important cellular mechanism that regulates memory formation. Long-term potentiation (LTP) and long-term depression (LTD) have been the primary models used to study the cellular and molecular basis of synaptic plasticity. LTP and LTD are modulated by a balance between kinases and phosphatases. LTP after synaptic stimulation often results from a postsynaptic Ca2+ influx and the activation of protein kinases, including mitogen-activated protein kinase (MAPK), calcium/calmodulin-dependent protein kinase II (CaMKII) and protein kinase A (PKA). In contrast, phosphatases such as PP2A, PP1 and PP2B that are present in the postsynaptic compartment are essential in the regulation of LTD.

Wip1 belongs to the protein phosphatase 2C (PP2C) family and is expressed in the brain. The expression of Wip1 is induced by a variety of stresses, such as ionising radiation (IR), ultraviolet (UV) radiation, and inflammatory cytokines. Once induced, Wip1 binds to and dephosphorylates several key signaling proteins involved in stress response. Identified targets of Wip1, such as p53, p38, H2AX and ATM, play vital roles in stress responses. Recently, we reported that Wip1 could regulate the morphology of dendritic spines. This observation indicates that Wip1 may play important roles in the central nervous system. Considering the important function of phosphatases in synaptic plasticity, we use Wip1 phosphatase knockout mice (Wip1/−/− mice) to explore the function of Wip1 phosphatase in synaptic plasticity.

CaMKII is a serine/threonine kinase that functions as a major mediator of LTP. It is highly expressed in the brain and is enriched at excitatory synapses and post-synaptic densities (PSD). Once activated by calcium/calmodulin, CaMKII is autophosphorylated at T286/287, which causes CaMKII activity to persist after the calcium concentration falls to baseline levels. This autophosphorylation of CaMKII is important for the LTP process, as the introduction of active CaMKII subunits into CA1 neurons causes large EPSC, and LTP can...
no longer be induced.\textsuperscript{17,18} Moreover, if autophosphorylation of CaMKII is prevented by a knock-in mutation of T286A CaMKII, LTP induction is prevented, and the mice show memory impairments.\textsuperscript{19} Interestingly, the transgenic mice that express constitutively activated CaMKII (T286D) show impaired spatial memory.\textsuperscript{20,21} Additionally, cellular transfection of T286D causes decreased excitatory postsynaptic currents (EPSCs).\textsuperscript{17} Considering that T305 phosphorylation and aberrantly phosphorylated CaMKII has been believed to be important in the LTP process, we measured LTD in Wip1\textsuperscript{−/−} mice (111.1 ± 7.6%, n = 8) and CaMKII phosphatase modulates dephosphorylation of CaMKII

**Results**

**Suppressed LTP in the hippocampus CA1 region of adult Wip1\textsuperscript{−/−} mice**

To investigate whether Wip1 modulates synaptic plasticity, we measured LTP expression in the hippocampus CA1 area of 3-month-old Wip1\textsuperscript{−/−}, Wip1\textsuperscript{+/−} and Wip1\textsuperscript{+/+} mice. LTP was induced by \( \theta \) burst stimuli (TBS; 4 trains of 10 bursts of 4 pulses with 20 s, 200 ms, and 10 ms intervals between trains, bursts, and pulses, respectively) in the CA1 region of all 3 groups of mice. As shown in Figure 1A, LTP was successfully established in Wip1\textsuperscript{−/−} mice (148.8 ± 7.0%, n = 8) and Wip1\textsuperscript{+/−} mice (149.5 ± 7.6%, n = 8) but was reduced in Wip1\textsuperscript{+/+} mice (111.1 ± 3.0%, n = 8). Repeated measures 2-way ANOVA revealed that LTP was significantly suppressed in the hippocampus of Wip1\textsuperscript{−/−} mice, (group: F (2,924) = 8.7; the interaction of group \( \times \) time: F (88,924) = 3.9, \( P < 0.01 \); post-hoc Bonferroni tests: \( P < 0.01 \) for comparison of fEPSP slopes 50–60 min after TBS application, Wip1\textsuperscript{−/−} vs. Wip1\textsuperscript{+/+}). No unequivocal difference was found between Wip1\textsuperscript{−/−} mice and Wip1\textsuperscript{+/+} mice. The LTP data derived from these groups are summarised and compared in Figure 1B. Thus, the deletion of Wip1 leads to a decrease of hippocampal LTP.

**Aberrant CaMKII phosphorylation level in the hippocampus of Wip1\textsuperscript{−/−} mice**

To explore possible downstream element(s) of Wip1 during synaptic plasticity, we compared the phosphorylation level of several kinases involved in the LTP process. The phosphorylation levels of CaMKII, AKT, GSK3\( \beta \), and ERK in the hippocampus of adult Wip1\textsuperscript{−/−} mice and their Wip1\textsuperscript{+/+} littermates were tested. Interestingly, western blot analysis showed that the phosphorylation levels of CaMKII \( \alpha \) and \( \beta \) at the Thr 286/287 site (Fig. 2A and B) were significantly increased in Wip1\textsuperscript{−/−} mice compared to their Wip1\textsuperscript{+/+} littermates. Western blot also showed that the phosphorylation levels of AKT, GSK3\( \beta \), and ERK were unchanged (Fig. 2C and D). As Thr305 phosphorylation determines the effect of T286 phosphorylation on synaptic plasticity,\textsuperscript{17} we also examined the phosphorylation level of CaMKII T305. The results showed that there was also a significant increase in T305 phosphorylation in Wip1\textsuperscript{−/−} mice (Fig. 2A and B). Together, these data show that Wip1 deficiency causes aberrant phosphorylation of CaMKII at T286 and T305.

**Wip1 phosphatase modulates dephosphorylation of CaMKII**

Wip1 is a member of the protein phosphatase 2C (PP2C) family,\textsuperscript{7} and PP2C dephosphorylates CaMKII at both T286/287 and T305/306.\textsuperscript{27} Thus, we investigated whether Wip1 could dephosphorylate CaMKII. Immunoblotting showed that CaMKII and Wip1 were colocalised in cultured hippocampal neurons (Fig. 3A), suggesting that Wip1 is co-expressed with CaMKII in hippocampal neurons. To further confirm that Wip1 phosphatase could dephosphorylate CaMKII, we over expressed Wip1 and CaMKII\( \beta \) cDNA for 24 h. Western blot showed that Wip1 expression was significantly increased, whereas phosphorylation of both CaMKII T286 and T305 was decreased (Fig. 3B). These observations demonstrated that Wip1 modulates CaMKII dephosphorylation. To confirm whether there is a physical interaction between Wip1 and CaMKII, immunoprecipitation was performed using hippocampal lysates. The results showed that there was a physical interaction between CaMKII and Wip1 in the hippocampus of wild type mice but not in Wip1\textsuperscript{−/−} mice.
mice (Fig. 3C and D). These observations suggest that Wip1 modulates the dephosphorylation of CaMKII.

**LTD is facilitated in the hippocampus CA1 region of adult Wip1−/− mice**

As aberrant elevated CaMKII T286/287 and T305 phosphorylation also regulate the LTD process,17,26 we further measured LTD in the hippocampus of Wip1−/− mice. We measured LTD expression in the hippocampus CA1 area of 3-month-old Wip1−/− mice, Wip1+/− mice and Wip1+/+ mice. LTD was induced by low frequency stimulation (LFS, 900 pulses at 1 Hz) in the CA1 region of all 3 groups of mice. As shown in Figure 4A, LTD expression was significantly facilitated in the Wip1−/− mice (64.2 ± 6.5%, n = 8) compared to Wip1+/+ mice.
Figure 3. Wip1 phosphatase modulates CaMKII dephosphorylation. (A) Triple immunostaining for CaMKII (red), Wip1 (green), and DAPI (blue) in cultured hippocampal neurons showed that Wip1 is colocalised with CaMKII in hippocampal neurons. Scale bar is 50 μm. (B) HEK293 cells were transfected with a CaMKIIβ construct and a control vector or with increasing amounts of Wip1 cDNA. The levels of Wip1, p-CaMKIIβ (Thr286), p-CaMKIIβ (Thr305) and CaMKII were analyzed via immunoblotting. (C and D) Hippocampal lysates from Wip1+/− mice and Wip1−/− mice were immunoprecipitated with anti-Wip1 (C), anti-CaMKII (D) or an IgG control. Immune precipitates were analyzed via immunoblotting using antibodies against CaMKII (C) and Wip1 (D).
(87.3 ± 7.4%, n = 8) and Wip1+/− mice (83.3 ± 6.4%, n = 8). Repeated measures 2-way ANOVA revealed that LTD was significantly facilitated in the hippocampus of Wip1−/− mice (group: F (2,819) = 2.9; the interaction of group x time: F (78,819) = 1.7, P < 0.01; post hoc Bonferroni tests: P < 0.05 for comparison of fEPSP slopes 50–60 min after LFS application, Wip1−/− vs. Wip1+/+). No unequivocal difference was found between Wip1+/− mice and Wip1+/+ mice. The LTD data derived from these groups are summarised and compared in Figure 4B. This result indicates that Wip1 deletion causes an enhancement of hippocampal LTD.

**Aberrant elevation of CaMKII phosphorylation is responsible for abnormal GluR1 membrane distribution in Wip1-null mice**

CaMKII elicits hippocampal LTP through regulating the distribution of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA) receptors. AMPA receptors transportation from recycling endosomes to the plasma membrane, which is an important mechanism underlying the regulation of synaptic plasticity. Therefore, we investigated whether Wip1 signaling could modulate the membrane expression of AMPA and NMDA receptors. We extracted membrane proteins from the hippocampal slices of Wip1−/− mice and from their wild-type littersmates. Western blot using antibodies against GluR1, GluR2, NR2A and NR2B showed that GluR1 membrane expression was significantly increased in Wip1−/− mice compared to Wip1+/+ mice (P < 0.01) (Fig. 5A and B). In addition, the total levels of GluR1, GluR2, NR2A and NR2B were indistinguishable between Wip1+/+ and Wip1−/− mice. Thus, Wip1 deficiency causes an abnormal GluR1 membrane distribution.

To support the notion that aberrant elevation of CaMKII phosphorylation is responsible for the abnormal GluR1 distribution in Wip1−/− mice, Wip1−/− hippocampal slices were pre-incubated with a vehicle or the CaMKII inhibitor Myr-AIP. Notably, following AIP treatment, the abnormal membrane expression of GluR1 in Wip1−/− mice was reversed (Fig. 5C and D), indicating that Wip1/ CaMKII signaling modulates the distribution of GluR1.

**Discussion**

In the present study, we first found that Wip1−/− mice exhibit suppressed hippocampal LTP and an aberrant elevation of CaMKII T286/287 and T305 phosphorylation. As phosphorylation of CaMKII at these sites also regulates the LTD process, we then measured LTD in Wip1−/− mice and observed an elevation of hippocampal LTD in Wip1−/− mice. Furthermore, we demonstrated that Wip1 deficiency-induced aberrant CaMKII phosphorylation is responsible for the abnormal GluR1 membrane distribution in Wip1−/− mice. Taken together, our results show that Wip1−/− mice exhibit suppressed LTD and facilitated LTD. Moreover, the aberrant elevation of CaMKII phosphorylation caused by an abnormal GluR1 distribution is associated with the abnormal synaptic plasticity in Wip1−/− mice.

**A new phosphatase responsible for learning and plasticity**

Protein phosphorylation has been considered as a key element in hippocampus-based synaptic plasticity. Although many studies have focused on the activation of kinases involved in synaptic plasticity, protein phosphatases have an equally dynamic and
critical role for the induction of both LTP and LTD.\textsuperscript{5} Protein phosphatase-1 (PP1) has been shown to gate LTP and must be inhibited for LTP induction and expression.\textsuperscript{30–32} Additionally, PP1 and protein phosphatase 2 (PP2A) are required for LTD.\textsuperscript{33} PP2A can be activated by a Ca\textsuperscript{2+}-dependent process, but its inhibition is required both in LTP induction and maintenance.\textsuperscript{34,35} Mice that express a constitutively active protein phosphatase 2 (PP2B, Calcineurin) exhibit an inhibition of LTP,\textsuperscript{36} and a specific PP2B inhibitor can facilitate LTP.\textsuperscript{37} In this study, we provided genetic evidence that Wip1 phosphatase is essential for hippocampus-dependent synaptic plasticity. A deficiency of Wip1 suppresses LTP and facilitates LTD. However, how synaptic stimulation regulates Wip1 phosphatase is still not clear. In vitro research showed that Wip1 is consistently phosphorylated by kinases and maintained at a low level via proteasomal degradation in unstimulated cells.\textsuperscript{38} If Wip1 is also regulated by phosphorylation in synaptic plasticity, there may exist a Wip1/CaMKII switch-facilitator similar to PP1/CaMKII\textsuperscript{39} or PP2A/CaMKII.\textsuperscript{35} These phosphatase/kinase switches are believed to be important in regulating synaptic plasticity. Further quantitative studies on these switches are required to provide a better understanding of how Wip1 is regulated in synaptic plasticity.

\textbf{Wip1 deficiency-induced aberrant CaMKII phosphorylation disrupts hippocampus-dependent synaptic plasticity}

CaMKII has been firmly connected to synaptic plasticity since its discovery.\textsuperscript{15,40} Activation of CaMKII by Ca\textsuperscript{2+} influx via NMDA receptors is essential for normal hippocampal LTP and hippocampus-based learning.\textsuperscript{14,40} The phosphorylation of CaMKII at Thr286/287 is important in LTP induction, and mutations that prevent the autophosphorylation of this site result in LTP deficits.\textsuperscript{19} Additionally, the phosphorylation of CaMKII T305/306 is believed to reduce the autonomous kinase activity.\textsuperscript{23,24} Our results showed that in Wip1\textsuperscript{¡/¡} mice, aberrant elevations of CaMKII phosphorylation at both T286/287 and T305 were associated with a disruption of synaptic plasticity. This is a confusing but acceptable result. Recent studies have revealed possible functions of CaMKII in the LTD process.\textsuperscript{17,26} Other reports have also suggested that an elevation of CaMKII T286 phosphorylation may not favor LTP. Although low expression level of CaMKII phosphorylation facilitates LTP, mice with high constitutively active CaMKII (T286D) showed deficits in LTP.\textsuperscript{41} Another study showed that autonomously active CaMKII (T286D) fails to induce LTP but causes an LTD-like weakening.\textsuperscript{20,21} Similar to our results, the Angleman Syndrome mouse model exhibits LTP deficits...
due to the elevation of T286 and T305 but a reduction in CaMKII activity and autophosphorylation. Likewise, hyperactivated CaMKII was found to be associated with learning impairments in attention-deficit/hyperactivity disorder (ADHD) models and mice models of thalassemia X-linked mental retardation (ATRX) (Shioda N et al. 2011). These reports indicate that aberrantly elevated CaMKII T286/287 phosphorylation may impair LTP. Additionally, CaMKII T305/T306 phosphorylation shows inhibitory effects to CaMKII activity and could cause a shift toward LTD. Protein serine/threonine phosphatases that act on CaMKII T286/287 have putative roles in modulating CaMKII-dependent synaptic plasticity. PP1, PP2A, and PP2C were able to dephosphorylate CaMKII in vitro. Here, we have shown that Wip1, as a member of the PP2C family, also modulates the dephosphorylation of CaMKII T286/287 and T305/306, as Wip1-deficient mice exhibit aberrant elevated CaMKII phosphorylation at both T286/287 and T305. Aberrant elevation of CaMKII phosphorylation is believed to be associated with several mental diseases, such as ATRX, ADHD and Angelman syndrome. Although these studies did not identify the exact phosphatase, a reduction of protein phosphatase activities may play an essential role in the elevated CaMKII activity and accompanying behavioral deficits. Future explorations of the involvement of Wip1 phosphatase in these mental disorders would be interesting.

**Wip1 deficiency causes abnormal GluR1 membrane distribution**

The number of synaptic AMPARs is an important factor during plasticity. We have observed an abnormal increase in GluR1 membrane expression in the hippocampus of Wip1−/− mice. GluR1 recycling is crucial for maintaining a mobile population of membrane AMPARs that can be delivered to synapses to increase synaptic strength. However, as the membrane insertion of GluR1 is significantly increased in Wip1−/− mice, there is not enough available recycled GluR1 to respond to synaptic stimulation, and as a result, synaptic plasticity is impaired. CaMKII-mediated GluR1 trafficking is one of the most important mechanisms for increasing the number of synaptic AMPARs. Interestingly, recent studies have shown that during the LTD process, CaMKII decreases the expression of membrane GluR1. Thus, CaMKII phosphorylation is required for both LTP and LTD. The downstream mechanisms for such differences are unclear but likely include secondary effects on CaMKII regulation, such as substrate site selection and inhibitory phosphorylation at T305/306. In our study, we showed that Wip1 deficiency-induced CaMKII T286/287 and T305 elevations were associated with an abnormal GluR1 distribution. Furthermore, we found that the abnormal GluR1 distribution was reversed by inhibiting CaMKII. Although the detailed mechanism is not clear, our results indicated an involvement of Wip1 phosphatase in the CaMKII-mediated GluR1 distribution in synaptic plasticity.

In conclusion, this study provides significant new insights into the function of Wip1 phosphatase for modulating LTP and LTD. Wip1 deletion-induced aberrant elevation of CaMKII phosphorylation potentially underlies synaptic plasticity impairment. Moreover, we showed that Wip1 phosphatase could modulate the dephosphorylation of CaMKII, indicating that Wip1 is a new phosphatase that regulates CaMKII dephosphorylation in synaptic plasticity processes. We also found that in Wip1−/− mice, aberrantly elevated CaMKII phosphorylation induced an abnormal membrane expression of GluR1, which further led to an impairment of GluR1 recycling during synaptic plasticity.

**Materials and methods**

**Mouse strains**

Male C57BL/6 mice were used in this study. Animals were maintained under a 12 h/12 h light/dark cycle with the light on at 07:00 AM. They were fed standard chow and water ad libitum. The ambient temperature and relative humidity were maintained at 22 ± 2°C and 55 ± 5%, respectively. All experiments were conducted under the institutional guidelines of the Animal Ethics Committee, Kunming Medical University.

Wip1−/− mice (C57BL/6 background) were a kind gift from Professor Lawrence A. Donehower. Genotyping of the Wip1−/− mice was carried out using PCR analysis on tail genomic DNA with the following primers:

- **BD1**: GACAGCTCCTGTGGGATAATGCT
- **BD2**: GGTGACTTGTGTTGTTTAGA
- **BD3**: GCAGGGTGGTTGTTGCT
- **BD4**: GCATGCTCCAGACTGCTT

**Antibodies**

Rabbit anti-CaMKII (pan), rabbit anti-CaMKII (Thr286/287) and mouse anti-β-actin were purchased from Abcam. Mouse anti-AKT, rabbit anti-AKT (Thr308), rabbit anti-GSK3β, rabbit anti-p-GSK3β (Ser9), rabbit anti-ERK (pan) and rabbit anti-p-ERK (Thr202/Tyr204) were purchased from Cell Signaling Technology. Rabbit anti-CaMKII (Thr305), rabbit anti-GluR1, rabbit anti-GluR2, rabbit anti-NR2A and mouse anti-NR2B were purchased from Millipore. Rabbit anti-Wip1 was purchased from Abcam.
purchased from Cell Signaling Technology and Bethyl Laboratories.

**Primary hippocampal neuron culture**

Hippocampal neurons were prepared from 18-day mouse embryos, as previously reported. The cells were plated on 24-well poly-D-lysine-coated glass slides in neurobasal media containing 2% B27 supplement (Invitrogen), L-glutamine (0.5 mM) and 0.1 mg/ml penicillin/streptomycin solution (Invitrogen) for 7 d

**Immunocytochemistry**

Neurons were washed in phosphate buffered saline (PBS) and fixed in 4% paraformaldehyde for 20 minutes. After washing in PBS, cells were blocked with 10% normal goat serum in PBS plus 0.3% Triton for one hour. The cells were incubated with primary antibody overnight at 4°C, washed 3 times using PBS, and incubated with Alexa Fluor 488 or 555 conjugated secondary antibody (Invitrogen) for 1 hour at room temperature. 4, 6-Diamidino-2-phenylindole (DAPI) was added as a nuclei counterstaining dye.

**Hippocampal slice preparation**

Two- to 3-month-old male mice were anaesthetised and decapitated. Acute hippocampal slices were coronally cut 400 μm thick using a vibratome (WPI). The slices were gently transferred to a submersion holding chamber containing ACSF (mM: NaCl 126, KCl 2.5, NaH2PO4 1, CaCl2 2.5, MgSO4 1.5, NaHCO3 26, and glucose 10) bubbled with 95% O2 and 5% CO2. The slices were recovered at room temperature for at least 1 h before being transferred to a submersion recording chamber (PSMI; Harvard Apparatus, Holliston, MA, USA), in which the slices were continually perfused with oxygenated ACSF at a rate of 1–2 ml/min at room temperature.

**Hippocampal slice electrophysiological recording**

Field excitatory postsynaptic potentials (fEPSPs) were recorded in the stratum radiatum of the hippocampus CA1 region using a glass microelectrode filled with 3 M NaCl (resistance 1–4 MΩ). The Schaffer collateral pathway was stimulated with concentric bipolar electrodes (FHC Corporate and Manufacturing, Bowdoin, ME, USA). Baseline fEPSPs were recorded at 0.033 Hz with a stimulating strength adjusted to yield approximately 50% of the maximal response. After stable baseline responses were achieved for at least 30 min, LTD was induced by delivering 900 pulses at low frequency (1 Hz) stimulation (LFS). Theta burst stimuli (TBS; 4 trains of 10 bursts of 4 pulses with 20 s, 200 ms, and 10 ms intervals between trains, bursts, and pulses, respectively) were applied to the slices to induce LTP. The electrophysiological data were acquired using a multiclamp 700 A amplifier (Axon instruments, Molecular Devices, USA), filtered at 0.1–5 kHz, digitised at 10 kHz, and analyzed with Clampfit version 10.0 (Axon Instruments, USA). The analyzed data were further processed using Origin 5.1 (Microcal Software Northampton, MA).

**Drug application**

All drugs were applied after dissolving to the desired final concentrations in ACSF. The effective CaMKII inhibitor, Myristoylated AIP (Myr-AIP), was purchased from Sigma. The compound was dissolved in dimethylsulfoxide (DMSO) stock solution and stored at −20°C until the day of the experiment. The concentration of DMSO in the perfusing medium was 0.05% in control experiments; this DMSO concentration had no effect on EPSPs. The CaMKII inhibitor Myr-AIP (1 μM) was used at its final concentration in ACSF, which is sufficient for its function according to previous studies.

**Membrane protein extraction**

Membrane proteins were extracted from hippocampal slices of adult male C57BL/6 mice using a Mem-PER Eukaryotic Membrane Protein Extraction Kit (Thermo Scientific). Briefly, hippocampal slices were lysed with a detergent, after which a second detergent was added to solubilise the membrane proteins. The cocktail was incubated at 37°C for 30 min. The hydrophobic protein fraction was separated from the hydrophilic protein fraction through phase partitioning. For SDS-PAGE, the membrane protein fraction was diluted 2- to 5-fold to prevent band and lane distortion caused by high concentrations of the detergent.

**Western blot assay**

Hippocampal slices or neuronal samples were homogenized in RIPA buffer (150 mM NaCl, 1.0% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris-HCL, pH 8.0) containing a protease inhibitor and a phosphatase inhibitor (Thermo). The lysates were then dissolved in 2× laemml sample buffer (Biorad) and boiled at 95°C for 5 minutes. For protein gel blot, lysates (20 μl) were added to SDS-PAGE and electroblotted onto PVDF membranes. The membranes were blocked using 5% skim milk in TBS-T and probed with antibodies of interest. Super Signal West Dura Extended Duration...
Substrate (Pierce) was used to reveal the antibody-antigen complexes. For proteins of the same size, we loaded the same amount of lysates from the same samples to different membranes. For statistical analyses, we first normalized the expression of each protein to its own β-actin on the same membrane.

**Cell line culture and plasmids transfection**

The HEK293 cell line was cultured in DMEM (Life Technologies) containing 10% foetal calf serum (Gibco) and maintained at 37°C with 5% CO2. The cell line was obtained from ATCC. Plasmids were transfected using lipofectamine2000 (Life Technologies) according to the manufacturer’s instructions. The Wip1 and CaMKII/β constructs were purchased from Origene.

**Immunoprecipitation**

Tissues were lysed in 0.5% (v/v) Nonidet P40 lysis buffer supplemented with a protease inhibitor cocktail and PhosStop (Roche). The total cell extracts were incubated with specific antibodies and precipitated with protein A/G sepharose beads (sigma) before washing and suspension in Laemmli. The lysates were then stored for western blot assays.

**Data analysis**

All values are expressed as the mean ± SEM, where n indicates the number of slices. One-way ANOVA was performed to determine whether there were significant differences followed by a post-hoc Bonferroni test; P < 0.05 indicated a significant difference. The electrophysiological data were acquired using an Axon multi-clamp 700 A amplifer (Molecular Devices Corp., Sunnyvale, CA, USA), filtered at 0.1–5 kHz, digitised at 10 kHz, and analyzed using pClamp8.1 software (Molecular Devices Corp).

**Abbreviations**

AIP | Autocamtide−2–Related Inhibitory Peptide  
AMPA | α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate  
CaMKII | Ca^{2+}/calmodulin-dependent protein kinase II  
LTD | long-term depression  
LTP | long-term potentiation  
NMDA | N-Methyl-D-aspartic acid  
PKA | Protein kinase A  
Wip1 | Wild type p53 induced phosphatase gene

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**Disclosure of potential conflicts of interest**

No potential conflicts of interest were disclosed.

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**Author contributions**

Z. X., Z. H., W. H. designed research; Z. H., W. H. performed research; Z. H., W. H., M. Z., H. Z., Z. Y., Q. M., and D. X. analyzed data; Z. X., Z. H. wrote the paper.
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