Deficiency of AMPAR–Palmitoylation Aggravates Seizure Susceptibility

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Synaptic AMPAR expression controls the strength of excitatory synaptic transmission and plasticity. An excess of synaptic AMPARs leads to epilepsy in response to seizure-inducible stimulation. The appropriate regulation of AMPARs plays a crucial role in the maintenance of the excitatory/inhibitory synaptic balance; however, the detailed mechanisms underlying epilepsy remain unclear. Our previous studies have revealed that a key modification of AMPAR trafficking to and from postsynaptic membranes is the reversible, posttranslational S-palmitoylation at the C-termini of receptors. To clarify the role of palmitoylation-dependent regulation of AMPARs *in vivo*, we generated GluA1 palmitoylation-deficient (Cys811 to Ser substitution) knock-in mice. These mutant male mice showed elevated seizure susceptibility and seizure-induced neuronal activity without impairments in synaptic transmission, gross brain structure, or behavior at the basal level. Disruption of the palmitoylation site was accompanied by upregulated GluA1 phosphorylation at Ser831, but not at Ser845, in the hippocampus and increased GluA1 protein expression in the cortex. Furthermore, GluA1 palmitoylation suppressed excessive spine enlargement above a certain size after LTP. Our findings indicate that an abnormality in GluA1 palmitoylation can lead to hyperexcitability in the cerebrum, which negatively affects the maintenance of network stability, resulting in epileptic seizures.

Key words: AMPAR; palmitoylation; GluA, seizure; epilepsy

Significance Statement

AMPA receptors predominantly mediate excitatory synaptic transmission. AMPARs are regulated in a posttranslational, palmitoylation-dependent manner in excitatory synapses of the mammalian brain. Reversible palmitoylation dynamically controls synaptic expression and intracellular trafficking of the receptors. Here, we generated GluA1 palmitoylation-deficient knock-in mice to clarify the role of AMPAR palmitoylation *in vivo*. We showed that an abnormality in GluA1 palmitoylation led to hyperexcitability, resulting in epileptic seizure. This is the first identification of a specific palmitoylated protein critical for the seizure-suppressing process. Our data also provide insight into how predicted receptors such as AMPARs can effectively preserve network stability in the brain. Furthermore, these findings help to define novel key targets for developing anti-epileptic drugs.

Introduction

Glutamate is the major excitatory neurotransmitter in the mammalian CNS. Among the ionotropic glutamate receptors

(iGluRs), AMPA-type receptors mediate the majority of the fast component of EPSCs (Seeburg, 1993; Hollmann and Heinemann, 1994). The expression of postsynaptic AMPARs is closely

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linked to excitatory synaptic strength (Shepherd and Huganir, 2007; Heine et al., 2008). Therefore, quantitative control of synaptic AMPAR number is critical for basal synaptic transmission, mammalian synaptic plasticity, and higher brain function (Collingridge et al., 2004; Shepherd and Huganir, 2007; Kessels and Malinow, 2009; Huganir and Nicoll, 2013). The AMPAR is composed of four subunits, GluA1, GluA2, GluA3, and GluA4 (also known as GluR1–4, GluRA–D, or GluR α 1–4). The subunit composition of AMPARs determines the routes of AMPAR trafficking, so GluA1 is dominant over others during activity-dependent AMPAR exocytosis to synapses. GluA2 is the primary determinant during endocytosis from synapses (Anggono and Huganir, 2012). In these processes, AMPAR trafficking to and from synapses is dynamically regulated by posttranslational protein modifications (Jiang et al., 2006; Anggono and Huganir, 2012; Lussier et al., 2015). Many studies have shown that the protein phosphorylation of AMPAR subunits plays a critical role in the regulation of synaptic plasticity, such as LTP and LTD or hyperexcitability (Rakhade et al., 2012). AMPAR phosphorylation modulates the properties of AMPAR ion channels and the membrane trafficking of AMPARs to the postsynaptic membrane (Malinow and Malenka, 2002; Derkach et al., 2007; Shepherd and Huganir, 2007).

Another key modification of AMPARs is the covalent attachment of the lipid palmitate at their intracellular cysteine residues via thioester bonds. This process of posttranslational protein S-palmitoylation is labile and reversible, like phosphorylation (Resh, 2006; Linder and Deschenes, 2007; Fukata and Fukata, 2010). Generally, palmitoylation acts as a sticky tag that can direct proteins, including many neuronal receptors and ion channels, to specific regions of the plasma membrane or specific intracellular membranes or vesicles (Kang et al., 2008; Shipston, 2011; Borroni et al., 2016). We have previously reported that palmitoylation regulates the synaptic expression and localization of AMPARs (Hayashi et al., 2005; Lin et al., 2009; Thomas et al., 2012, 2013; Thomas and Hayashi, 2013). The mammalian AMPAR subunits GluA1–4 are palmitoylated at two distinct sites: the transmembrane (TMD) 2 and C-terminal domains (Hayashi et al., 2005; Hayashi, 2014). TMD 2 palmitoylation causes an accumulation of receptors in the Golgi apparatus, which appears to act as a “quality control” mechanism to ensure correct receptor maturation. In contrast, the C-terminal palmitoylation site on AMPARs is implicated in the “quantitative regulation” of synaptic receptor number, which is associated with complex neuronal events. Palmitoylation on the C-terminal domain inhibits GluA1 interaction with the 4.1N protein, which stabilizes AMPAR expression on the cell surface and regulates endocytosis and AMPAR insertion in LTP (Shen et al., 2000; Hayashi et al., 2005; Lin et al., 2009). Furthermore, palmitoylation and depalmitoylation of the AMPAR is regulated in an activity-dependent manner (Hayashi et al., 2005; Kang et al., 2008), which is induced by cocaine or a high-fat diet (Van Dolah et al., 2011; Spinelli et al., 2017). In summary, palmitoylation appears uniquely suited to create dynamic control of synaptic expression and intracellular trafficking

of AMPARs. Studies have revealed the importance of posttranslational AMPAR palmitoylation; however, its role is yet to be elucidated *in vivo*.

Here, we generated mice lacking the C-terminal palmitoylation site of GluA1 at Cys811 (C811) and demonstrated that a deficiency in palmitoylation enhanced seizure susceptibility and LTP-induced spine enlargements without affecting gross brain structure and normal excitatory synaptic transmission in knock-in homozygote mice. Our findings presented here indicate that an abnormality in palmitoylation-dependent regulation of the AMPARs may lead to hyperexcitability that reduces the maintenance of network stability, resulting in epileptic seizures.

Materials and Methods

Generation of palmitoylation-deficient GluA1CS mice and experimental animals. To construct the targeting vector, we identified a bacterial artificial chromosome (BAC) clone RP24-223G10 (BACPAC Resources Center; RRID:SCR_007215) prepared from the C57BL/6 strain containing exon 16 of GluA1 using basic local alignment search tool searches against the mouse genome sequence database. The targeting vector comprised 5′-side (5.3 kb) and 3′-side (6.3 kb) arms with PGK–neomycin-resistant (neo) flanked by two loxP sites and the diphtheria toxin gene (DT). The 3′-side arm contained exon 16 with the palmitoylation site Cys811 mutated to serine. In addition, a silent NruI site mutation was introduced just after the serine mutation site to distinguish the mutant and WT alleles. All mutation procedures were performed by QuikChange II site directed mutagenesis kit (Stratagene; RRID:SCR_013575). These fragments were introduced into the pDEST-DT by Red/ET recombination using BAC subcloning kit (Gene Bridges; RRID:SCR_000483). The targeting vector was linearized by NotI and electroporated into embryonic stem (ES) cell line RENKA derived from C57BL/6N strain, as described previously (Mishina and Sakimura, 2007). Then, G-418-resistant clones were picked as positive clones and confirmed by Southern blot hybridization analysis using outer probes. PCR-amplified 231 and 243 bp fragments and 635 bp PstI fragments from pLNeo were used as 5′, 3′, and neo probes, respectively. The 5′ and 3′ probes were amplified by PCR using 5′-GGCCAGAAGGGGAGGTA GCC-3′ and 5′-AGGACATCGGCTGGCACTGA-3′, 5′-TGGCGTT GGAGTGAGTGTC-3′ and 5′-TAAATCCATGATTCAGACA-3′ as primers, respectively. Recombinant ES cells were injected into eight-cell stage embryos of the CD-1 mouse strain. The embryos were cultured to blastocysts and transferred to a pseudopregnant CD-1 mouse uterus. The resulting chimera mice were mated to C57BL/6N mice to yield heterozygous [GluA1 WT/cysteine to serine (CS)–Neo(+)] mice. These mice were crossed to CAG-Cre (backcrossed six times to C57BL/6) to remove the *neo* cassette from the germline through *Cre/loxP*-mediated excision. After confirmation of *neo* excision by Southern blot analysis and PCR, homozygotes CS/CS mice were obtained by crossing heterozygous GluA1 WT/CS pairs. Mutants with no *Cre* gene were used for subsequent breeding. The mutant mice were backcrossed onto the C57BL/6N strain at least five times. The GluA1 CS allele was identified by PCR using the primers 5′-CTCTGAGCCTGAGCAATGTG-3′ and 5′-CTGCCTTC TGCTGTGTTCAA-3′. The intercross of heterozygotes resulted in the production of WT, heterozygous, and homozygous offspring at the expected 1:2:1 Mendelian ratio. Only male mice were used for subsequent analyses.

C57BL/6N mice were obtained from Charles River Laboratories. CAG-Cre mice were a kind gift from Dr. J. Miyazaki. Thy1-yellow fluorescent protein (YFP)-H transgenic mice were purchased from the Jackson Laboratory and backcrossed onto the C57BL/6J strain 15 times, followed by the C57BL/6N strain at least eight times. Mice were fed with standard laboratory chow and water in standard animal cages under a 12 h light/dark cycle.

All animal care and experiments were performed in accordance with the regulations and institutional guidelines of Niigata University, the University of Tokyo, the National Center of Neurology and Psychiatry (NCNP), the Japanese Pharmacological Society, and the Japan Neuroscience Society. The technical protocols for animal experiments in this

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study were approved by the institutional review committees of the Graduate School of Medicine, the University of Tokyo, and National Institute of Neuroscience, NCNP.

Drugs and antibodies. Pentylentetrazole (PTZ), valproic acid sodium salt (VPA), phenobarbital (PB), and diazepam (DZP) were purchased from Sigma-Aldrich; RRID:SCR_008988. Anti-GluA1 (ab31232, Abcam; RRID:AB_2113447), anti-GluA1 phospho-Ser831 (04-823, Millipore; RRID:AB_1977218), anti-GluA1 phospho-Ser845 (04-1073, Millipore; RRID:AB_1977219), anti-GluA2 (MAB397, Millipore; RRID:AB_2113875), anti-GluA2/3 (07-598, Millipore; RRID:AB_310741), anti-GluN1 (ab17345, Abcam; RRID:AB_776808), anti-GluN2A (AB1555P, Millipore; RRID:AB_90770), anti-GluN2B (ab65783, Abcam; RRID:AB_1658870), anti-PSD-95 (MA1-045, Thermo Fisher Scientific; RRID:AB_325399), anti-GAPDH (5174, Cell signaling technology; RRID:AB_10622025), anti-c-Fos (C-10, Santa Cruz Biotechnology; RRID:AB_10610067), and anti-Arc (OP1/2MBL-2012B and OP2-2012, H.O.) antibodies were used for the experiments.

Biochemical analysis. Hippocampi or cortices were directly lysed in SDS sample buffer. Lysates were separated by SDS-PAGE followed by Western blotting with each antibody. Reactive bands were visualized with the ECL Prime Western Blotting Detection System and chemiluminescent images were acquired using the ImageQuant LAS 4000 mini imager (GE Healthcare; RRID:SCR_000004). Palmitoylation of AMPAR subunits, NMDAR subunits, and scaffolding MAGUK proteins were assessed using the acyl-biotinyl exchange (ABE) method (Drisdell et al., 2006; Kang et al., 2008), as described previously (Wan et al., 2007). Briefly, brain samples were directly denatured in lysis buffer containing 25 mM HEPES, pH 7.4, 150 mM NaCl, 2% SDS, 5 mM EDTA, protease inhibitor mixture (Roche), and 20 mM methyl methanethiosulfonate (MMTS) to block free thiols and acetone precipitation was used to move between steps. Following lysis, excess MMTS was removed by acetone precipitation and pellets were resuspended in buffer containing 4% (w/v) SDS buffer (4SB). Samples were diluted and incubated for 1 h in either 0.7 M hydroxylamine (NH₂OH, pH 7.4) to cleave thioester bonds or 50 mM Tris, pH 7.4. After acetone precipitation to remove hydroxylamine or Tris, pellets were resuspended in 4SB and incubated for 1 h in 50 mM Tris, pH 7.4, containing 0.2 mM sulfhydryl-reactive biotin-HPDP (Cayman Chemical; RRID:SCR_008945) at room temperature. To remove unreacted HPDP–biotin, acetone precipitations was performed and pellets were resuspended in 4SB. SDS was then diluted to 0.1% (w/v) and biotinylated proteins in the samples were affinity purified using Streptavidin Mag Sepharose (GE Healthcare; RRID:SCR_000004). SDS sample buffer was used to cleave HPDP–biotin and to release purified proteins from the beads. The released proteins in the supernatant were denatured in SDS sample buffer and processed for Western blotting.

Histology and immunohistochemistry. Brains were perfused and fixed with 4% (for Nissl staining and c-Fos expression) or 2% (Arc expression) paraformaldehyde (PFA) in PBS and transferred to 10%, 20%, and 30% sucrose in PBS solution step by step every 24 h. After tissues were embedded in optimal cutting temperature (OCT) compound, cryosections were cut at 20 μ m thickness unless otherwise noted. Sagittal or coronal sections were stained with cresyl violet and observed under the light microscope for Nissl staining or processed for immunohistochemistry using anti-c-Fos and anti-Arc antibodies with DAPI. Confocal z-stack images of dendritic segments (0.3–0.8 μ m intervals, 5–10 image sections/stack) acquired with the LSM710 using a 63 $\times$ objective (numerical aperture 1.4, oil) were projected onto single planes by summation.

Electrophysiology. Mouse brain slices were prepared as described previously with minor modifications (Zhao et al., 2011; Ting et al., 2014). Mice were anesthetized with sevoflurane and brains were quickly removed. Hippocampal slices were prepared using a linear slicer Pro 7 (Dosaka EM) in an ice-cold solution (in mM: 98 Choline-Cl, 2.5 KCl, 1.25 NaH₂PO₄, 30 NaHCO₃, 20 HEPES, 25 glucose, 2 thiourea, 5 Na-ascorbate, 2.5 Na-pyruvate, 0.5 CaCl₂, and 10 MgSO₄). After sectioning, the slices were recovered with the cutting medium for 10 min at 32°C and then stored for at least 1 h at room temperature in a solution containing the following (in mM): 98 NaCl, 2.5 KCl, 1.25 NaH₂PO₄, 30 NaHCO₃, 20 HEPES, 25 glucose, 2 thiourea, 5 Na-ascorbate, 2.5 Na-pyruvate, 2 CaCl₂, and 2 MgSO₄. Each slice was then transferred to the

recording chamber and perfused ($\sim$ 3 ml/min) with artificial CSF (ACSF) containing the following (in mM): 125 NaCl, 2.5 KCl, 26 NaHCO₃, 1.25 NaH₂PO₄, 4 CaCl₂, 4 MgSO₄ and 10 glucose at 28–32°C. All external solutions were equilibrated with 95% O₂ and 5% CO₂, pH 7.4. The electrophysiological signal was acquired using a MultiClamp 700B, DigiData 1440, and pClamp 10 (Molecular Devices; RRID:SCR_011323). Liquid junction potential and series resistance were left uncompensated. The series resistance was monitored frequently during recordings and neurons showing >30 M Ω and a large drift (20%) in series resistance were excluded from the analysis. For voltage-clamp recordings, patch pipettes were filled with Cs⁺-based intracellular solution containing the following (in mM): 135 CsMeSO₄, 5.0 TEA-Cl, 1 MgCl₂, 0.5 EGTA, 3.0 Mg-ATP, 0.3 Na-GTP, 10 Na-phosphocreatine, 2 QX314, and 10 HEPES, pH 7.2, and the resistance was 4–6 M Ω . For the recording of miniature EPSCs (mEPSCs), neurons were held at -70 mV in the presence of 1 μ M tetrodotoxin (TTX) and 100 μ M picrotoxin in the bath solution. The results were analyzed using pClamp 10 software (Molecular Devices; RRID:SCR_011323). A bipolar stimulating electrode (FHC) was used to evoke baseline EPSC of -200 to -400 pA at -70 mV. For measurement of AMPA/NMDA ratios, bath solution containing 100 μ M picrotoxin was used. AMPA/NMDA ratios were calculated as the ratio of peak current at -70 mV to the current at 80 ms after stimulus onset at $+40$ mV. For AMPAR rectification experiments, we added 100 μ M DL-AP5 and 100 μ M spermine to the external and internal solution, respectively. The rectification index was calculated as the ratio of peak current at -70 mV and $+50$ mV. For current-clamp recordings, patch pipettes were filled with a K⁺-based intracellular solution containing the following (in mM): 135 K-gluconate, 5.0 KCl, 1 MgCl₂, 0.5 EGTA, 3.0 Mg-ATP, 0.3 Na-GTP, 10 Na-phosphocreatine, and 10 HEPES, pH 7.2, and the resistance was 4–6 M Ω . To measure the ratio of EPSPs to IPSPs, the resting membrane potential of patched neurons was set to ~ -60 mV by current injection. Schaffer collaterals were stimulated using a bipolar electrode and the amplitudes of evoked EPSPs were adjusted to ~ 10 mV. The EPSP/IPSP ratio was calculated as the maximum EPSP amplitude divided by the IPSP amplitude.

Chemical LTP (cLTP). cLTP was induced as described previously with some modifications (Liao et al., 2001; Lu et al., 2001; Passafium et al., 2001; Park et al., 2004; Kopec et al., 2006, 2007). Briefly, 400 μ m hippocampal slices were prepared from 3- to 4-week-old Thy1-YFP (WT) or GluA1 CS/CS; Thy1-YFP (CS/CS) mice in the cutting buffer containing the following (in mM): 20 HEPES, 30 NaHCO₃, 1.25 NaH₂PO₄, 10 MgSO₄, 2.5 KCl, 0.5 CaCl₂, 160 sucrose, 25 D-glucose, 2.5 sodium pyruvate, 2.5 sodium ascorbate, and 1 N-acetyl-L-cysteine (NAC), pH 7.4, on ice. Then, slices were recovered in the incubation buffer containing the following (in mM): 20 HEPES, 30 NaHCO₃, 1.25 NaH₂PO₄, 100 NaCl, 2.5 KCl, 2 CaCl₂, 2 MgSO₄, 25 D-glucose, 2.5 sodium pyruvate, 2.5 sodium ascorbate, and 1 NAC, pH 7.4, at room temperature for 1–2 h, followed by the incubation in ACSF containing the following (in mM): 26 NaHCO₃, 1 NaH₂PO₄, 125 NaCl, 4.4 KCl, 2.5 CaCl₂, 1.5 MgSO₄, and 10 D-glucose at 30°C for 30 min. cLTP induction was performed in Mg²⁺-free ACSF containing the following (in mM): 26 NaHCO₃, 1.2 KH₂PO₄, 124 NaCl, 4 CaCl₂, 3 KCl, and 10 D-glucose with 200 μ M glycine and 100 μ M picrotoxin at 30°C for 30 min. Baseline controls were acquired in Mg²⁺-free ACSF with 200 μ M glycine, 100 μ M picrotoxin, and 100 μ M DL-AP5, a NMDAR blocker, at 30°C for 30 min incubation. All solutions were continuously aerated with 5% CO₂ and 95% O₂ bubbling. Slices were fixed with 4% PFA in PBS for overnight and transferred to 30% sucrose in PBS solution. After the tissue was embedded in OCT compound, cryosections were recut at 30 μ m thickness. Spine volumes were quantified as described previously (Matsuzaki et al., 2001, 2004). Background signals were subtracted from the projected images and YFP fluorescence signals showing spine morphology on clearly separated apical dendrites of CA1 pyramidal neurons were selected for blind analysis (see “Histology and immunohistochemistry” section). For intensity comparisons, mean fluorescent intensities and the area of individual spines were measured using ImageJ (RRID:SCR_003070).

Seizure observation and kindling procedure. PTZ was administered intraperitoneally at a dose of 20–80 mg/kg. We used the PTZ-induced kindling model as described previously with some modifications

(Schröder et al., 1993; Becker et al., 1995). Briefly, C57BL/6N and CS/CS mice were intraperitoneally injected with PTZ (30 mg/kg) once every 48 h for 12 total injections and mice showing more than three consecutive stage 4 seizures were used as kindled mice. Control mice were injected with PBS. Phenobarbital or diazepam was administered intraperitoneally for the indicated periods before PTZ injection. We examined seizure events during the 20 min observation period after injection. Seizure intensity was scored as follows: stage 0, no response; stage 1, ear, mouth, and facial twitching; stage 2, head nodding and convulsive twitching axially through the body; stage 3, limbic myoclonus; stage 4, rearing demonstrated by standing on hind legs and wild jumping; stage 5, generalized tonic-clonic seizures; and stage 6, death. Two-way repeated-measures ANOVA followed by Fisher's LSD test was used for the kindling experiment and the Mann–Whitney test was used to compare two groups.

Experimental design and statistical analysis. The design of each experiment and statistical analysis methods used were described in each of the experimental sections. Statistical analyses were performed using Prism 6 (GraphPad Software; RRID:SCR_002798) and Excel (Microsoft; RRID:SCR_016137). All data are expressed as mean $\pm$ SEM unless indicated otherwise.

Results

Generation of GluA1 C-terminal palmitoylation mutant mice

To elucidate directly the role of GluA1 palmitoylation in the regulation of synaptic function *in vivo*, we analyzed a line of GluA1 non-palmitoylation mutant mice that lack the C-terminal palmitoylation site. Mice were generated by introducing a mutation at C811 in the GluA1 gene using homologous recombination techniques (knock-in). A targeting vector encoding CS substitution at C811 at exon 16 was constructed with a loxP-flanked *neo* marker at intron 15 (Fig. 1A and Fig. 1-1A, available at <https://doi.org/10.1523/JNEUROSCI.1590-18.2018.f1-1>). Correctly targeted ES cells containing the C811S mutation and *neo* cassette were injected into blastocysts. Chimera mice carrying the mutant allele were bred to C57BL/6N mice to generate heterozygous mice [GluA1 WT/CS-Neo(+)]. Heterozygous mice were then bred to CAG-Cre mice to delete the *neo* cassette from the germline via the Cre-loxP system (GluA1 WT/CS) and then intercrossed to produce homozygous mice (GluA1 CS/CS, hereinafter called CS/CS). The success of these procedures was confirmed by Southern blot and PCR analysis (Fig. 1-1B–D, available at <https://doi.org/10.1523/JNEUROSCI.1590-18.2018.f1-1>). Both heterozygous and homozygous GluA1 C811S mutant mice appeared to grow normally, have healthy characteristics, breed normally, and have similar fertility to WT littermate mice.

First, we confirmed that the mutation of the palmitoylation site by ABE assay using specific antibodies (Fig. 1B). Decreased levels of GluA1 palmitoylation were found in the whole brain of CS/CS mice ($68.9 \pm 1.9\%$ compared with WT control, $n = 3$ mice, respectively; $p < 0.001$; t test; Fig. 1C). The residual signals likely represented palmitoylation at Cys585 on TMD 2, which is intact in CS/CS mice. The palmitoylation of other AMPAR GluA2/GluA3 subunits and the major synaptic scaffolding protein, PSD95, were unaffected in CS/CS mice, which ensured that GluA1 C811 palmitoylation was specifically absent without affecting other palmitoylated synaptic proteins. A non-palmitoylated synaptic scaffolding protein, SAP102, was used to determine background level of palmitoylation. In addition, Nissl-stained brain sections from CS/CS mice did not show any gross abnormalities in cytoarchitecture compared with WT mice (Fig. 1D and Fig. 1-1E, available at <https://doi.org/10.1523/JNEUROSCI.1590-18.2018.f1-1>). Together, these results indicate that the lack of GluA1 C811 palmitoylation site does not alter brain gross anatomy or general synaptic palmitoylation.

Augmented GluA1 phosphorylation at Ser 831 and increased protein expression of GluA1 in GluA1 C811S mutant mice

Next, we examined the protein expression and phosphorylation of the major AMPAR subunits, GluA1, GluA2, and GluA3; the iGluR NMDAR subunits GluN1, GluN2A, and GluN2B (also called NR1/GluR ζ 1, NR2A/GluR ϵ 1, and NR2B/GluR ϵ 2); and PSD95 in the adult hippocampus. Western blot analysis showed that there was no significant difference in expression in these synaptic proteins between WT and CS/CS mice (Fig. 2A). Furthermore, GluA1 phosphorylation was increased at Ser831 (S831), but not Ser845, in CS/CS mice (Fig. 2B). At several phosphorylation sites in GluA1, phosphorylation at S831 is increased by LTP induction (Jiang et al., 2006; Derkach et al., 2007; Shepherd and Huganir, 2007; Kessels and Malinow, 2009; Lussier et al., 2015). The expression level of GluA1 was slightly increased in CS/CS mice, but this was not statistically different ($107.9 \pm 16.5\%$ compared with WT, $n = 8$ mice, respectively; $p = 0.20$; t test). GluA1 protein expression was increased in the cortex of adult CS/CS mice compared with WT mice (Fig. 2C). These data suggest that GluA1 function is consistently upregulated in the cerebrum of CS/CS mice.

Normal synaptic transmission in GluA1 C811S mutant mice

Next, we examined basal synaptic transmission mediated by AMPARs in CS/CS mice. To characterize basal synaptic properties, we performed a series of electrophysiological experiments using acute hippocampal slices. First, the input–output relationships of field EPSPs (fEPSPs) were analyzed by the extracellular field recording at hippocampal CA3–CA1 synapses (Fig. 3A). The results did not show any difference in the input–output relationship between WT and CS/CS littermate mice (between genotypes: $F_{(1,28)} = 0.88$, $p = 0.36$; two-way repeated-measures ANOVA). In addition, paired-pulse facilitation, an index of presynaptic function, was also measured (Fig. 3B). We found no obvious changes between WT and CS/CS mice in the paired-pulse ratio (between genotypes: $F_{(1,28)} = 3.39$, $p = 0.08$; two-way repeated-measures ANOVA). These results indicated that basal synaptic strength and presynaptic release function of CA3–CA1 synapses was almost normal in CS/CS mice. To further explore any potential changes in basal synaptic transmission, we measured AMPAR-mediated miniature EPSCs (mEPSCs) by whole-cell patch-clamp recordings (Fig. 3C) and found no significant differences in mean amplitude or frequency of mEPSCs (amplitude: WT, 13.62 ± 1.06 pA, CS/CS, 15.00 ± 0.87 pA; frequency: WT 0.79 ± 0.08 Hz, CS/CS 0.80 ± 0.15 Hz; $n = 15$ cells from 4 WT mice, $n = 13$ cells from 4 CS/CS mice). Next, the ratio of AMPAR to NMDAR-mediated synaptic currents were measured to test whether the GluA1 C811S mutation led to alterations in NMDAR-mediated synaptic transmission (Fig. 3D). The results showed that there was no statistical difference between WT and CS/CS mice (9.90 ± 2.00 , $n = 19$ cells from 4 WT mice; 9.12 ± 2.26 , $n = 19$ cells from 4 CS/CS mice). To estimate the composition of calcium-permeable AMPARs and GluA1 homotetramers, we finally analyzed the current–voltage (I – V) relationship of AMPAR-mediated currents (Fig. 3E). CS/CS mice display identical I – V curves and similar rectification index to WT mice (0.33 ± 0.03 , $n = 13$ cells from 4 WT mice; 0.31 ± 0.03 , $n = 11$ cells from 4 CS/CS mice), which is typically reflected by the similar ratio of presence of the GluA1 homotetramers and GluA1/GluA2 heterotetramers composition. Collectively, these results suggest that the possible upregulation of GluA1 observed in CS/CS mice does not cause abnormalities in the basal synaptic transmission or membrane properties of CA1 pyramidal neurons.

Figure 1. Generation of GluA1 C-terminal palmitoylation deficient mice. **A**, Top, Schematic representation of GluA1 protein structure. Black bars indicate four transmembrane domains. Exon 16 encoding the C-terminal palmitoylation site (C811) is shown. Middle, Genomic DNA structure with the relevant restriction enzyme sites; E, EcoRI; S, Scal. White boxes represent exon 15 and 16. Bottom, Targeting vector and targeted genes. The Ser substitution for Cys811 (CS) is marked in red. Neo-loxP, PGK promoter, neomycin-resistance gene with loxP sequence at both sides; arrows, PCR primers; N, silent NruI site. The 5' and 3' probes for Southern blot analysis, which contain the genomic sequence outside of the targeting vector, are depicted as black bars. **B**, Schematics of the ABE assay. The ABE assay consists of four steps, as follows: (1) blocking of free thiol (sulfhydryl groups, SH) on Cys residues by MMTS; (2) chemical cleavage of thioester linkage with hydroxylamine (NH_2OH); (3) specific labeling with biotin-conjugated SH attacking reagent; and (4) purification of biotin-labeled (corresponding to palmitoylated) proteins with streptavidin-conjugated beads. **C**, Reduced palmitoylation at Cys811 in GluA1 CS/CS mice. Representative blots of protein expression and palmitoylation in the cerebrum lysate prepared from WT and GluA1 CS/CS (CS/CS) mice (left) showed that palmitoylation of GluA1, but not of other proteins, was specifically reduced in CS/CS mice. Experiments were performed in triplicate ($n = 3$) and (Figure legend continues.)

Figure 2. Protein expression and phosphorylation of iGluR subunits in the hippocampus and cortex of GluA1 C811S mutant mice. **A**, Protein expression of AMPAR subunits, NMDAR subunits, and PSD-95 in hippocampal lysates from WT (white bars) and CS/CS (black bars) mice. Typical representative blots (left) and protein expression levels are shown (right, WT: $n = 8$, CS/CS: $n = 8$). **B**, GluA1 phosphorylation at S831 and S845 in hippocampus from WT (white bars) and CS/CS (black bars) mice. Typical blots are shown as representative samples (left). Phosphorylation levels are compared with WT mice (middle) and those normalized to GluA1 protein expression are shown (right) (WT: $n = 8$, CS/CS: $n = 8$). **C**, Protein expression levels of AMPAR subunits, NMDAR subunits, and PSD-95 in cortical lysates from WT (white bars) and CS/CS (black bars) mice. Typical blots as representative (left) and protein expression levels are shown (right, WT: $n = 8$, CS/CS: $n = 8$). Error bars indicate SEM. * $p < 0.05$, t test.

(Fig. 3-1, available at <https://doi.org/10.1523/JNEUROSCI.1590-18.2018.f3-1>). Moreover, the GluA1 subunit lacking the C811 palmitoylation site was properly targeted to synapses, indicating that palmitoylation at C811 may not be critical for maintaining the steady-state level of receptors at synapses.

Disturbed chemical LTP-induced spine volume enlargements in GluA1 C811S mutant mice

To assess whether a CS mutation affected neural circuits, we measured the synaptic excitatory/inhibitory (E/I) balance in acute hippocampal slices at the basal level. Schaffer collateral fibers were stimulated and the evoked EPSP–IPSP sequences were recorded in CA1 pyramidal neurons (Fig. 4A). As depicted, we could not find any difference in the EPSP/IPSP ratio between WT and CS/CS mice (4.40 ± 0.63 , $n = 14$ cells from 3 WT mice; 3.09 ± 0.60 , $n = 17$ cells from 3 CS/CS mice, $p = 0.15$ by t test).

To further examine the physiological role of GluA1 C811 palmitoylation, we tested whether the GluA1 C811S mutation affected synaptic plasticity in CA1 pyramidal neurons. Our simple electrophysiological stimulation (100 pulses at 100 Hz $\times$ 4 trains)

(Figure legend continued.) palmitoylation levels were analyzed using two approaches: percentage of recovery (right, top) and normalized levels to WT mice (right, bottom). White bars are WT mice and black bars are CS/CS mice. Error bars indicate SEM. *** $p < 0.001$, t test. **D**, Nissl-stained sections across the hippocampus and cortex from WT or CS/CS mice showing no gross abnormalities in the cytoarchitecture (left: typical coronal sections; right: typical sagittal sections). Scale bar, 1 mm. See also Extended Figure 1-1, available at <https://doi.org/10.1523/JNEUROSCI.1590-18.2018.f1-1>.

Figure 3. Normal basal synaptic transmission in GluA1 C811S mutant mice. **A**, Input–output relationship. fEPSPs were recorded at different stimulation intensities by extracellular field recordings (left). To compare the input–output relationship, fEPSP slopes are plotted against stimulation intensities (right) (WT: $n = 15$ slices from 5 mice, CS/CS: $n = 15$ slices from 5 mice). **B**, Paired-pulse ratio (PPR). Responses to paired-pulse stimulation using different inter stimulus intervals were recorded (left). PPRs are plotted against interstimulus intervals (right) (WT: $n = 15$ slices from 5 mice, CS/CS: $n = 15$ slices from 5 mice). **C**, mEPSCs. CA1 pyramidal neurons were held at -70 mV and mEPSCs were recorded in the presence of picrotoxin and TTX (left). Amplitude and frequency of mEPSCs are shown (middle and right) (WT: $n = 15$ cells from 4 mice, CS/CS: $n = 13$ slices from 4 mice). **D**, AMPA/NMDA ratio. CA1 pyramidal neurons were held at either -70 mV or $+40$ mV and EPSCs evoked by the stimulation of Schaffer collateral were recorded (left). AMPA/NMDA ratios are calculated as described in experimental procedures (middle). Cumulative distributions of the AMPA/NMDA ratio are also shown (right) (WT: $n = 19$ cells from 4 mice, CS/CS: $n = 19$ slices from 4 mice). **E**, Rectification of AMPAR-EPSCs. Evoked AMPAR-EPSCs were recorded at various holding potentials (-70 , -50 , -30 , -10 , $+10$, $+30$, and $+50$ mV) (left). Normalized $I-V$ plots are made from peak amplitude of AMPAR-EPSCs at various potential (middle). Rectification index are also plotted (right) (WT: $n = 13$ cells from 4 mice, CS/CS: $n = 11$ slices from 4 mice). See also Extended Figure 3-1, available at <https://doi.org/10.1523/JNEUROSCI.1590-18.2018.f3-1>.

Figure 4. Normal LTP induction in GluA1 C811S mutant mice. **A**, E/I balances in CA1 pyramidal neurons. Representative traces of compound PSP in WT (left) and CS/CS (middle) mice are shown. The EPSP/IPSP ratios were calculated and plotted (right) (WT: $n = 14$ slices from 3 mice; CS/CS: $n = 17$ slices from 3 mice). **B**, Hippocampal CA3–CA1 LTP was induced using 4 trains of high-frequency stimulation (each train includes 100 Hz for 1 s, the intertrain interval is 10 s). fEPSP slopes (normalized to percentage of baseline) were plotted over time (left). Representative traces of fEPSPs before and after LTP induction are also shown. Scale bars, 0.5 mV, 10 ms. The summary plot of LTP (averaged for the last 10 min) level is shown in the right (WT: $n = 11$ slices from 5 mice, CS/CS: $n = 11$ slices from 5 mice). Error bars indicate SEM. **C**, Hippocampal CA3–CA1 LTD was induced by the bath application of 20 μ M NMDA for 3 min. The normalized fEPSP slopes were plotted over time (left). Representative traces of fEPSP before and after chem-LTD induction are also shown. Scale bars, 0.5 mV, 10 ms. The summary plot of chem-LTD (averaged for last 10 min) level is shown on the right (WT: $n = 11$ slices from 5 mice, CS/CS: $n = 11$ slices from 5 mice). Error bars indicate SEM.

failed to detect a difference in LTP induction between WT and CS/CS mice (Fig. 4B, $182.8 \pm 9.6\%$, $n = 11$ slices from 5 WT mice; $184.9 \pm 15.6\%$, $n = 11$ slices from 5 CS/CS mice, $p = 0.91$ by t test). In addition, NMDA ($20 \mu\text{M}$, 3 min)-induced LTD showed no significant difference between WT and CS/CS mice (Fig. 4C, $62.5 \pm 6.8\%$, $n = 11$ slices from 5 WT mice; $68.0 \pm 10.1\%$, $n = 11$ slices from 5 CS/CS mice, $p = 0.66$ by t test).

Many studies have revealed a positive correlation among AMPAR currents, AMPAR synaptic expression, and spine volume (Holtmaat and Svoboda, 2009; Kasai et al., 2010). Lines of evidence indicate that volume expansion of dendritic spines highly correlates with LTP as well as surface expression of AMPARs. Previous studies have shown that the green fluorescence protein and its spectral variants are useful tools to analyze spine structures in transfected neurons. Thy1-YFP-H transgenic mice show sparse subset labeling in excitatory neurons (Feng et al., 2000). Therefore, we visualized spine structures in 3- 4-week-old Thy1-YFP (WT) mice or GluA1 CS/CS; Thy1-YFP (CS/CS) mice by observing endogenous YFP fluorescent signals and quantified stimulation-dependent changes of spine volume on the apical dendrites of CA1 pyramidal neurons in their hippocampal acute slices (Fig. 5A).

In excitatory synapses, NMDARs are the main triggers in the induction of synaptic plasticity (Mori and Mishina, 2003; Collingridge et al., 2004; Lau and Zukin, 2007; Bliss and Collingridge, 2013). Here, we used the selective activation of synaptic NMDARs to induce cLTP by applying the NMDAR co-agonist glycine and the GABA_A receptor antagonist picrotoxin (Fig. 5A). This glycine-induced cLTP protocol has previously been demonstrated to cause a rapid exocytosis of AMPARs into the synaptic plasma membrane, leading to LTP of AMPAR-mediated excitatory transmission (Liao et al., 2001; Lu et al., 2001; Passafaro et al., 2001; Park et al., 2004). Spine volumes showed no significant difference between WT and CS/CS slices in their basal states (Fig. 5B and Fig. 5-1, control, available at <https://doi.org/10.1523/JNEUROSCI.1590-18.2018.f5-1>). Upon cLTP induction, spine enlargements were observed in both WT and CS/CS groups at 30 min after glycine application, but the spine volume distributions were significantly differed between the groups; CS/CS slices showed larger spine volume compared with WT slices (Fig. 5B and Fig. 5-1, cLTP, available at <https://doi.org/10.1523/JNEUROSCI.1590-18.2018.f5-1>). Interestingly, the difference between WT and CS/CS groups under the cLTP condition was mostly found in a larger half of population, whereas there seemed to be no difference in the smaller half (Fig. 5B, $p = 0.033$ by Kolmogorov–Smirnov test). Indeed, when we compared the spine volume of the third quartile (Q3) in each dendritic segment analyzed, the CS/CS group was significantly larger than WT under the cLTP condition, whereas there was no difference between the WT and CS/CS groups (Fig. 5C, ANOVA with *post hoc* Sidak's multiple comparisons). cLTP stimulation caused significant enlargement in both

Figure 5. Abnormal cLTP in GluA1 C811S mutant mice. **A**, Typical images of apical dendrites in CA1 pyramidal neurons, prepared from Thy1-YFP (WT) or GluA1 CS/CS; Thy1-YFP (CS/CS) mice with or without cLTP induction. Scale bar, $7.5 \mu\text{m}$. **B**, Cumulative frequency of cLTP-induced spine volume enhancements [WT (control): $n = 229$ spines on 12 dendrites, $n = 8$ mice, [CS/CS (control): $n = 236$ spines on 9 dendrites, $n = 8$ mice, [WT (cLTP): $n = 188$ spines on 9 dendrites, $n = 8$ mice, [CS/CS (cLTP): $n = 193$ spines on 8 dendrites, $n = 8$ mice, $*p < 0.05$, Kolmogorov–Smirnov test]. **C**, Comparison of the spine volume of the third quartile (Q3) in each dendritic segment. [WT (control): $n = 12$ dendrites, [CS/CS (control): $n = 9$ dendrites, [WT (cLTP): $n = 9$ dendrites, [CS/CS (cLTP): $n = 8$ dendrites. Error bars indicate SEM. $*p < 0.05$, $****p < 0.0001$, ANOVA ($F_{3,34} = 17.96$, $p < 0.0001$) with Sidak *post hoc* multiple comparisons. See also Extended Figure 5-1, available at <https://doi.org/10.1523/JNEUROSCI.1590-18.2018.f5-1>.

groups. These findings suggested that the GluA1 palmitoylation function to suppress excessive spine enlargement above a certain size in the normal brain when excitatory neurons receive strong inputs, such as epileptic seizure-inducing stimulation.

Elevated seizure susceptibility in GluA1 C811S mutant mice

Our previous studies have revealed that depalmitoylation at GluA1 C811 suppresses stimulation-induced internalization of the AMPAR from the surface to intracellular regions in cultured cortical neurons (Hayashi et al., 2005; Lin et al., 2009). Therefore, we hypothesized that depalmitoylation of AMPARs would cause excess accumulation and stable localization of newly inserted AMPARs in excitatory synapses, which could form the basis of hyperexcitability *in vivo*. To test the possibility that CS/CS mice are prone to hyperexcitation, a noncompetitive GABA_A receptor antagonist, PTZ, was used to induce convulsions. Without PTZ administration, CS/CS mice showed no apparent epileptic manifestations like WT mice. Consistent with the literature, mice administered with a single low PTZ dose ($20\text{--}30 \text{ mg/kg}$) exhibited ear and facial twitching (stage 1, Racine score) or, at times, convulsive twitching axially through the body (stage 2). PTZ application at a dose of $40\text{--}80 \text{ mg/kg}$ induced a seizure that was characterized by limbic myoclonus (stage 3) and clonic and/or tonic convulsion, jumping, and wild running (stage 4 or 5) in young adults (7 weeks old) and 3-month-old (13 weeks) adult mice (Fig. 6A,B). There were no significant differences in the highest seizure score between WT and CS/CS mice at any dose during 20 min observation after intraperitoneal PTZ injection. However, repeated occurrence of clonic and/or tonic seizures was

Figure 6. Epileptic seizure and kindling evoked by treating mice with PTZ. **A**, Seizure scores in 7-week-old WT (white bars) and CS/CS (black bars) mice received a single injection of PTZ at a dose of 0, 20, 30, 40, 60, or 80 mg/kg. Six white bars [WT]: $n = 21, 12, 10, 13, 18, 16$, six black bars [CS/CS]: $n = 17, 13, 12, 15, 16, 16$ (left to right). **B**, Seizure scores in 13-week-old WT (white bars) and CS/CS (black bars) mice received a single injection of PTZ at a dose of 0, 20, 40, or 60 mg/kg. Four white bars [WT]: $n = 16, 13, 14, 14$, four black bars [CS/CS]: $n = 13, 14, 17, 16$ (left to right). **C**, Occurrence of generalized tonic-clonic seizures (stage 4 and 5) in 7-week-old WT (white bars) and CS/CS (black bars) mice that received a single injection of PTZ at a dose of 40 (right, $n = 13, 15$, respectively) or 60 (left, $n = 17, 16$, respectively) mg/kg. **D**, Occurrence of generalized tonic-clonic seizures (stage 4 and 5) in 13-week-old WT (white bars) and CS/CS (black bars) mice that received a single injection of PTZ at a dose of 40 (right, $n = 13, 15$, respectively) or 60 (left, $n = 13, 13$, respectively) mg/kg. **E**, Kindling evoked by treating mice repeatedly with PTZ. WT (white) and CS/CS (black) mice were administered PTZ 12 times at a dose of 30 mg/kg (squares, $n = 8$, respectively) or saline (PBS, circles, $n = 5$, respectively) from 7-week-old (genotype effect, $F_{(1,14)} = 8.571, p = 0.011$; kindling effect, $F_{(11,154)} = 24.06, p < 0.0001$). **F**, Injection times of PTZ to induce the initial generalized tonic-clonic seizures ($n = 8$, respectively). **G**, Injection times of PTZ to establish the kindling response ($n = 8$, respectively). **H**, Continuous kindling response ($n = 8$, respectively). Error bars indicate SEM. * $p < 0.05$, ** $p < 0.01$. See also Extended Figure 6-1, available at <https://doi.org/10.1523/JNEUROSCI.1590-18.2018.f6-1>.

increased in both 7- and 13-week-old CS/CS mice compared with WT mice in the same condition (repeated times of stage 4 and 5: Fig. 6C,D, repeated times of stage 5, and Fig. 6-1A,B, available at <https://doi.org/10.1523/JNEUROSCI.1590-18.2018.f6-1>).

The kindling experiment is a commonly used experimental animal model for the development of epilepsy in which induced sei-

zures gradually increase after repeated electrical or chemical stimulation of certain forebrain structures. To further clarify the relationship between GluA1 C811S mutation and stimulation-dependent hyperexcitation, repeated administration of low-dose PTZ (30 mg/kg)-induced kindled seizure was examined to evaluate the possible differences in seizure susceptibility (Fig. 6E). In the kin-

dling experiment, CS/CS mice showed that an early induction of the initial tonic-clonic seizure (Fig. 6F), early establishment of kindling (Fig. 6G), and longer continuous kindling responses (Fig. 6H). These pharmacological experiments suggested that the GluA1 C811S mutation led to a decreased seizure threshold, resulting in more severe generalized tonic-clonic and myoclonic seizures induced by PTZ.

Increased expression of immediate early genes (IEGs) in GluA1 C811S mutant mice

Activity-induced expression of the neuronal IEGs *c-fos* and *Arc* (also called *arg3.1*) were explored to monitor neuronal activity in the cerebrum (Okuno, 2011; Shepherd and Bear, 2011; West and Greenberg, 2011; Madabhushi and Kim, 2018). Basal protein expression of both *c-Fos* and *Arc* were comparably low in the hippocampal dentate gyrus of WT and CS/CS mice (*c-Fos*; Fig. 7A, *Arc*, B). PTZ-produced seizure was accompanied by increased expression of *c-Fos* and *Arc* proteins in the hippocampus 2 h after treatment (Ramírez-Amaya et al., 2005; Vazdarjanova et al., 2006; Minatohara et al., 2016). Compared with WT mice, CS/CS mice showed stronger dose-dependent induction of *c-Fos* and *Arc* expression in their hippocampi, especially at a dose of 60 mg/kg. These data suggest that GluA1 C811 depalmitoylation is related with stimulation-dependent hyperactivity in the hippocampus. Consistent with the previous reports, *Arc* proteins were mainly detected in the soma of hippocampal dentate granule cells 2 h after PTZ injection and later translocated across dendrites during subsequent time points (Fig. 8B,D). Similar to WT mice, stimulation-induced *Arc* expression overlapped with *c-Fos* in CS/CS mice (Fig. 8A,C). The basal expression and PTZ-induced protein expression of *c-Fos* were significantly increased at any dose in the cerebral cortex of CS/CS mice (Fig. 7C and 7-1A–C, available at <https://doi.org/10.1523/JNEUROSCI.1590-18.2018.f7-1>). This correlated with enhanced GluA1 protein expression in the cortex (Fig. 2C). Interestingly, administration of PTZ failed to increase activity-dependent production of *Arc* protein in the cortex (Fig. 7D and Fig. 7-1D–F, available at <https://doi.org/10.1523/JNEUROSCI.1590-18.2018.f7-1>).

Reduced effects of anticonvulsants in GluA1 C811S mutant mice

Our experimental data indicated stimulation-induced hyperexcitation in the cerebrum of CS/CS mice. Therefore, effectiveness evaluation was assessed, concerning three types of clinically used

Figure 7. PTZ-induced neuronal activity visualized by IEGs. WT (white bars) and CS/CS (black bars) mice received a single injection of PTZ at a dose of 0, 20, 40, or 60 mg/kg and fixed 2 h after PTZ administration. Brain sections were incubated with each antibody and DAPI. Typical images are shown as representative (left). Scale bar, 200 μ m. Ratio of *c-Fos* or *Arc*-positive to DAPI-positive cells are shown (right). White broken lines indicate the edge of lateral ventricle. **A**, *c-Fos* expression in the hippocampal dentate gyrus ($n = 5$, respectively, $F_{(7,32)} = 10.58$, $p < 0.0001$). **B**, *Arc* expression in the hippocampal dentate gyrus ($n = 6$, respectively, $F_{(7,40)} = 14.22$, $p < 0.0001$). **C**, *c-Fos* expression in the cortex ($n = 19$, respectively, $F_{(7,144)} = 12.44$, $p < 0.0001$). **D**, *Arc* expression in the cortex ($n = 12$, respectively, $F_{(7,88)} = 3.166$, $p = 0.0049$). Error bars indicate SEM. ANOVA with Tukey *post hoc* test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared with WT controls. See also Extended Figure 7-1, available at <https://doi.org/10.1523/JNEUROSCI.1590-18.2018.f7-1>.

anticonvulsants, VPA, PB, and DZP, on PTZ-induced seizure in CS/CS mice. At first, we confirmed that administration of these anticonvulsants potently and dose-dependently suppressed the PTZ-induced tonic-clonic and myoclonic seizures in WT mice (Fig. 9).

Figure 8. PTZ-induced c-Fos and Arc proteins in hippocampal dentate gyrus. c-Fos (red) or Arc (green) expression with DAPI staining (blue) in WT and CS/CS mice 4 h after a single injection of seizure-inducing PTZ (60 mg/kg) or PBS (0 mg/kg). Typical images are shown as representative. Scale bar, 200 μ m. **A**, c-Fos expression with or without PTZ stimulation. **B**, Arc expression with or without PTZ stimulation. **C**, Merged image of c-Fos and Arc expression. **D**, Enlarged images of **B**.

VPA acts as an anticonvulsant to inhibit GABA transaminase, leading to an increase in the concentration of the inhibitory neurotransmitter, GABA. Next, the dose dependency of VPA-induced effects with 60 mg/kg PTZ-induced seizure was examined (Fig. 9*A,B*). Compared with WT mice, 30 min pretreatment with VPA exhibited a reduced effect on CS/CS mice in the highest seizure score during the 20 min observation (Fig. 9*A*) and repetitions of tonic-clonic seizures induced by PTZ (Fig. 9*B*). In addition, time courses of the pretreatment effect with VPA administration on the occurrence of 60 mg/kg PTZ-induced seizure scores were examined (300 mg/kg, Fig. 9-1*A*, available at <https://doi.org/10.1523/JNEUROSCI.1590-18.2018.f9-1>; 400 mg/kg, Fig. 9-1*B*, available at <https://doi.org/10.1523/JNEUROSCI.1590-18.2018.f9-1>). These results showed that VPA could significantly suppress the PTZ-induced seizure in both WT and CS/CS mice, but notably had lower effects on CS/CS mice. Namely, the GluA1 C811S mutation significantly reduced the anticonvulsive effects of VPA.

PB possesses anti-seizure activity by increasing the GABA_AR channel open probability at inhibitory synapses. The dose dependency and time course of PB pretreatment on 60 mg/kg PTZ-induced seizures were tested and the highest seizure scores are shown in Figure 9, *C–E*. Moreover, we observed the time courses

of pretreatment effects with PB on the occurrence of 60 mg/kg PTZ-induced tonic-clonic seizure (5 mg/kg PB, Fig. 9-1*C*, available at <https://doi.org/10.1523/JNEUROSCI.1590-18.2018.f9-1>; 10 mg/kg PB, Fig. 9-1*D*, available at <https://doi.org/10.1523/JNEUROSCI.1590-18.2018.f9-1>). These results showed that PB had reduced effects on PTZ-induced seizures in CS/CS mice compared with WT mice. Similarly, DZP potently and efficaciously blocked PTZ-induced seizure by prolonging GABA_AR channel open time. The dose dependency of DZP effects on 80 mg/kg PTZ-induced seizure was further tested. Pretreatment with DZP at doses of 1–5 mg/kg for 5 min showed lower effects on PTZ-induced seizure in CS/CS mice (the highest seizure score; Fig. 9*F*). DZP effects on the occurrence of PTZ-induced tonic-clonic seizure were relatively weak for CS/CS mice at all doses (repeated times of tonic-clonic seizure; Fig. 9*G*). These data are consistent with other results, implying that there exists increased seizure susceptibility in CS/CS mice.

Discussion

Epilepsy is characterized by recurring, unprovoked seizures, representing the abnormally synchronous activity of excitatory neu-

Figure 9. Inhibitory effects of anticonvulsants on PTZ-evoked seizure. **A**, Dose dependence of VPA. Seizure scores of 7-week-old WT (white bars) and GluA1 CS/CS (black bars) mice that received an administration of VPA at a dose of 0, 200, 300, or 400 mg/kg 30 min before a single injection of 60 mg/kg PTZ. Four white bars [WT]: $n = 19, 12, 10, 11$, four black bars [CS/CS]: $n = 19, 12, 11, 12$ (left to right). $F_{(7,98)} = 12.89, p < 0.0001$. **B**, Effects of VPA on occurrence of generalized tonic-clonic seizures. Four white bars [WT]: $n = 17, 11, 10, 11$, four black bars [CS/CS]: $n = 16, 7, 8, 10$ (left to right). $F_{(7,82)} = 11.91, p < 0.0001$. **C**, Dose dependence of PB. Seizure scores of 7-week-old WT (white bars) and GluA1 CS/CS (black bars) mice that received an administration of PB at a dose of 0, 5, or 10 mg/kg 30 min before a single injection of 60 mg/kg PTZ. Three white bars [WT]: $n = 19, 12, 14$, three black bars [CS/CS]: $n = 19, 12, 13$ (left to right). $F_{(5,83)} = 5.108, p = 0.0004$. **D**, Time course of PB effects at doses of 5 mg/kg. Seizure scores of 7-week-old WT (white squares) and CS/CS (black squares) mice that received an administration of PB at a dose of 5 mg/kg for the indicated periods before a single injection of 60 mg/kg PTZ. Four white squares [WT]: $n = 19, 10, 11, 12$, four black squares [CS/CS]: $n = 19, 13, 14, 12$ (left to right). $F_{(7,102)} = 3.556, p = 0.0018$. **E**, Time course of PB effects at doses of 10 mg/kg. Seizure scores of 7-week-old WT (white squares) and CS/CS (black squares) mice that received an administration of PB at a dose of 10 mg/kg for the indicated periods before a single injection of 60 mg/kg PTZ. Four white squares [WT]: $n = 19, 11, 14, 14$, four black squares [CS/CS]: $n = 19, 12, 10, 13$ (left to right). $F_{(7,104)} = 4.259, p = 0.0004$. **F**, Dose dependence of DZP. Seizure scores of 7-week-old WT (white bars) and CS/CS (black bars) mice that received an administration of DZP at a dose of 0, 0.1, 1, or 5 mg/kg, 5 min before a single injection of 80 mg/kg PTZ. Four white bars [WT]: $n = 16, 11, 10, 10$, four black bars [CS/CS]: $n = 16, 12, 13, 11$ (left to right). $F_{(7,91)} = 62.67, p < 0.0001$. **G**, Effects of DZP on occurrence of generalized tonic-clonic seizures. Four white bars [WT]: $n = 12, 8, 10, 10$, four black bars [CS/CS]: $n = 8, 8, 11, 11$ (left to right). $F_{(7,70)} = 19.80, p < 0.0001$. Error bars indicate SEM. ANOVA with Tukey–Kramer *post hoc* test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared with WT controls. See also Extended Figure 9–1, available at <https://doi.org/10.1523/JNEUROSCI.1590-18.2018.f9-1>.

rons in a focal area of the cerebrum and, in some cases, conveyed throughout the entire brain (Grone and Baraban, 2015; Staley, 2015). Epileptic seizure is associated with excessive cortical excitability resulting from imbalances between excitation and inhibition in the cerebrum. Disrupted E/I balance leads to perturbed

neuronal circuit function and repetitive seizures induce serious epilepsy (Paz and Huguenard, 2015). E/I imbalance is supposed to occur in very few excitatory synapses and then spreads to other areas. Although the appropriate regulation of ion channels and neurotransmitter receptors plays a crucial role in the mainte-

Figure 10. Model of the role of AMPAR palmitoylation *in vivo*. Palmitoylation of the GluA1 at C811 enables endocytosis of synaptic AMPARs and potentially upregulates the threshold of seizure occurrence in the cerebrum of WT mice (top). Constitutive depalmitoylation-mimicking C811S mutation of GluA1 prevents efficient internalization of synaptic AMPARs, which decreases the threshold of epileptic seizure when they receive seizure-inducing activity in the cerebrum of GluA1 CS/CS mice (bottom).

nance of the E/I synaptic balance (Haider and McCormick, 2009; Isaacson and Scanziani, 2011; Xue et al., 2014), detailed mechanisms underlining epilepsy remain unclear. Our findings presented here provide new insights into the regulation of functional AMPARs to preserve the stability of mammalian brain (Fig. 10).

The excitatory synapses throughout the cerebrum contain AMPARs to predominantly mediate synaptic transmission. Although many factors have been identified to regulate excitatory synaptic strength, previous research has revealed that one common mechanism is the control of the postsynaptic expression of AMPARs. In the present study, we demonstrated that a disruption of the GluA1 C-terminal palmitoylation site causes disturbed spine volume enhancements and increased seizure susceptibility without affecting gross brain structure. This non-palmitoylation mutation C811S did not exert an influence on excitatory glutamatergic synaptic transmission in the basal state (Fig. 3) and did not correlate with any behavioral defect of CS/CS mice. These data suggest that GluA1 palmitoylation at C811 in WT mice may be limited at the basal state. No significant change in intrinsic membrane excitabilities between WT and CS/CS mice indicates that there is no alteration in potassium channels (Fig. 3-1, available at <https://doi.org/10.1523/JNEUROSCI.1590-18.2018.f3-1>). Furthermore, no obvious E/I imbalance was observed in CS/CS mice at the basal level (Fig. 4A), suggesting that the C811S mutation did not affect GABAergic inputs and the formation of hippocampal circuits. These mutant mice showed gradually increased PTZ-induced generalized tonic-clonic and myoclonic seizure incidence and severity, indicating that GluA1 C811 palmitoylation properly controls the hyperexcitation threshold in the normal cerebrum (Fig. 6). In addition, the expression of the activity-regulated genes *c-fos* and *Arc* was augmented in response to tonic-clonic seizure-inducing PTZ in the hippocampus of CS/CS mice (Figs. 7, 8). Interestingly, in the neocortex, there was a discrepancy between *c-fos* and *Arc*; *c-fos* was upregulated by PTZ administration, whereas *Arc* was not, in

both WT and CS/CS mice. Such differential gene expression in the neocortex may represent a specific PTZ-driven activity pattern that preferentially upregulates a subset of activity-dependent genes (West and Greenberg, 2011; Madabhushi and Kim, 2018; Okuno et al., 2018; Tyssowski et al., 2018). In agreement with these pharmacological results, GluA1 phosphorylation at S831 in the hippocampus or AMPAR expression in the cortex was increased in CS/CS mice (Fig. 2). The GluA1 S831 phosphorylation is related with LTP (Jiang et al., 2006; Anggono and Huganir, 2012; Lussier et al., 2015). Therefore, these data strongly suggest that GluA1-containing AMPARs are ready to insert into hippocampal and cortical postsynapses in response to neuronal activities. Moreover, seizure blockade by clinically used anticonvulsants such as VPA, PB, and DZP showed reduced anti-epileptic properties in mutant mice when seizures are induced (Fig. 9). Our previous studies revealed that C811 palmitoylation of synaptic GluA1 is a critical driving force for AMPAR internalization from the surface (Hayashi et al., 2005). Our *in vivo* analysis presented here shows that the appropriate palmitoylation of AMPAR receptors prevents pathologic E/I imbalance due to excess excitatory synaptic transmission in normal brain. Concretely, the cLTP result suggests that palmitoylation deficiency induces exaggerated enlargement of relatively larger spines above a certain size, which possibly leads elevated seizure susceptibility (Fig. 5). Epileptic seizure may be aggravated when excitatory neuronal activities surpass a critical level in response to specific input, including these uncontrollably enlarged synapses. In contrast, significant difference was not observed in the lower, half-sized cLTP-induced spines. One interpretation is that some compensatory mechanisms may cover the impairment of AMPAR palmitoylation-dependent regulation in these smaller sized spines. Alternatively, AMPAR GluA1 subunits are dominantly palmitoylated in larger sized spines. At present, it remains unclear whether palmitoylation of GluA1 C811 occurs activity dependently *in vivo*. The molecular basis of dynamic regulation for GluA1 C811

palmitoylation should be further investigated in the future. Nevertheless, palmitoylation-dependent regulation of well balanced spine enlargement and newly inserted GluA1-containing AMPAR to synapses may ensure homeostatic fine tuning or adjustment to chronically suppress E/I imbalance (Davis, 2006; Marder and Goaillard, 2006; Heine et al., 2008; Turrigiano, 2011; Xue et al., 2014).

Although there are many factors related to the occurrence of epileptic seizures (Bateup et al., 2013; Noebels, 2015; Staley, 2015), our study provides a possibility that the palmitoylation-dependent appropriate regulation of synaptic AMPARs may suppress E/I imbalance, which prevents severe epileptic seizures. To date, major efforts to develop antiepileptic drugs targeting AMPARs have focused on the screening of useful antagonists for therapeutic use among a number of potential candidate compounds (Rakhade and Jensen, 2009; Rogawski, 2011; Russo et al., 2012). Perampanel (Fycompa), the only approved AMPAR antagonist for epilepsy, is clinically applied to treat patients with partial or generalized tonic-clonic seizures (Hibi et al., 2012; French et al., 2015; Kato et al., 2016; Faulkner, 2017). Presumably because AMPAR antagonists generally inhibit ion channel properties of all preexisting AMPARs on synapses in the whole brain, various adverse side effects are inevitable (Kramer et al., 2014; Zwart et al., 2014). Our present results suggest that AMPAR palmitoylation is a novel pharmacological target for treating refractory seizure in the future and compounds controlling AMPAR palmitoylation may have moderate side effects in various intractable epilepsies.

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