Supplementary Information for

Excitatory selective LTP of supramammillary glutamatergic/GABAergic co-transmission potentiates dentate granule cell firing

Eri Tabuchi, Takeshi Sakaba, Yuki Hashimotodani

Corresponding author: Yuki Hashimotodani
Email: yhashimo@mail.doshisha.ac.jp

This PDF file includes:

- Supplementary Materials and Methods
- SI References
- Figures S1 to S7
Supplementary Materials and Methods

Animals
We used C57BL/6 mice, VGluT2-Cre mice (Jackson labs, Slc17a6tm2(cre)Lowl/J, stock #016963) and VGAT-Venus mice (1) crossed with VGluT2-Cre mice (VGluT2-Cre/VGAT-Venus) of either sex for electrophysiological experiments. All animals were group housed in a temperature- and humidity-controlled room under a 12 hr light/12 hr dark cycle. Water and food were available ad libitum. Experiments were approved by the animal care and use committee of Doshisha University, and were performed in accordance with the guidelines of the committees.

Stereotaxic viral injections
Mice on postnatal days 19 to 20 were placed in a stereotaxic frame, and anesthetized with isoflurane (1.5–2.5%). A beveled glass capillary pipette connected to a microsyringe pump (UMP3, WPI) was used for viral injection. 200 nL of a virus-containing solution was injected into the SuM (relative to bregma, AP: −2.2 mm, ML: ±0.3 mm, DV: −4.85 mm) at a rate of 50 nL/min. The glass capillary was remained at the target site for 5 min before the beginning of the injection and was removed 10 min after infusion. VGluT2-Cre mice and VGluT2-Cre/VGAT-Venus mice were injected with AAV1.EF1a.DIO.hChR2(H134R)-eYFP (Addgene) or AAV1.EF1a.DIO.hChR2(H134R)-mCherry (Addgene), respectively.

Hippocampal slice preparation
Acute transverse hippocampal slices (300 µm thick) were prepared from mice 2–3 weeks after the injection of AAVs (5–6 weeks old). Mice were decapitated under isoflurane anesthesia. Briefly, the hippocampi were isolated, embedded in an agar block and cut using a vibratome (VT1200S, Leica microsystems) in an ice-cold cutting solution containing (in mM): 215 sucrose, 20 D-glucose, 2.5 KCl, 26 NaHCO3, 1.6 NaH2PO4, 1 CaCl2, 4 MgCl2, and 4 MgSO4. Brain blocks including the interbrain and midbrain were also isolated and fixed in 4% paraformaldehyde (PFA) for post hoc morphological analysis of the injection site. Hippocampal slices were transferred to an incubation chamber and incubated at 33.5°C in the cutting solution. After 30 min of incubation, the cutting solution was switched to an extracellular artificial cerebrospinal fluid (ACSF) containing (in mM): 124 NaCl, 2.5 KCl, 26 NaHCO3, 1 NaH2PO4, 2.5 CaCl2, 1.3 MgSO4 and 10 D-glucose at 33.5°C. Slices were subsequently kept at room temperature for at least 1 h before recording. Both the cutting solution and ACSF were oxygenated with
95% O$_2$ and 5% CO$_2$. After the recovery time, slices were transferred to a submersion-type recording chamber and perfused at 2 mL/min with oxygenated ACSF.

**Electrophysiology**

Whole-cell recordings were made from GCs, INs, and CA2 pyramidal neurons under an infrared differential interference contrast microscopy (IR-DIC, Olympus, BX51WI). For voltage-clamp recordings ($V_{\text{hold}} = -60$ mV), we used patch pipettes (3–6 MΩ) filled with an intracellular solution containing (in mM): 110 Cs-glucanate, 17.5 CsCl, 0.2 EGTA, 10 HEPES, 8 NaCl, 2 MgATP, 0.3 Na$_3$GTP, 10 phosphocreatine, pH 7.3 adjusted with CsOH (290–293 mOsm). In Fig. 4A, 20 mM Cs-glucanate was replaced with 20 mM BAPTA. For recordings of IPSCs (Fig. 2D and SI Appendix, Fig. S3A), CsCl was increased to 53 mM, and equimolar amount of Cs-glucanate was removed (calculated $E_{\text{Cl}} = -20$ mV).

We recorded from GCs with an input resistance of < 300 MΩ for mature GCs (2). For recordings from INs in the DG, we used VGAT-Venus BAC transgenic mice, in which Venus fluorescent proteins are expressed under the control of the VGAT promoter, enabling us to identify VGAT-expressing GABAergic neurons (1). VGAT-Venus mice were crossed with VGluT2-Cre mice for Cre-dependent expression of ChR2. INs were visually identified as Venus-expressing cells located at the border between the GC layer and the hilus, as reported previously (3). For recordings from CA2 pyramidal neurons, 0.5% biocytin was included in the intracellular solution for post hoc morphological analysis. CA2 pyramidal neurons were identified based on the location, size of the soma, and electrophysiological properties (firing properties and sag amplitude) (4, 5). ChR2-expressing SuM axons were activated at 0.05 Hz by a pulse of 470 nm blue light (5 ms duration, 10.5 mW/mm$^2$) delivered through a 40× objective attached to a microscope using an LED (Mightex or ThorLabs). For extracellular fiber stimulation, a patch pipette with a broken tip (with diameter of ~20–30 µm) filled with the ACSF was used. Depol-eLTP was typically induced by repeated postsynaptic depolarizations (2 s duration repeated 10 times every 5 s from a holding potential of ~60 mV to 0 mV). This depol-eLTP protocol was delivered within 20 min after whole-cell break-in. The pairing protocol (Fig. 1B and SI Appendix, Fig. S3B) was applied by 200 light pulses at 2 Hz paired with 100 s duration postsynaptic depolarization from -60 mV to 0 mV. For recordings of asynchronous synaptic events in the presence of strontium, ACSF was replaced by Ca$^{2+}$-free ACSF containing 8 mM SrCl$_2$. Asynchronous synaptic responses were evoked by light pulses at 0.1 Hz. After obtaining 10 min baseline, extracellular solution was replaced by normal ACSF containing 2.5 mM Ca$^{2+}$ to deliver the depol-eLTP protocol, and then extracellular solution was returned to Sr$^{2+}$-containing ACSF. To
test the effects of postsynaptic depolarization on NMDAR-oEPSCs (Fig. 2C), baseline responses were recorded with stimulus intensities yielding 30–50% of the maximum response to avoid putative saturation of NMDAR activation during the baseline period. For recordings of silent synapses, after obtaining oEPSCs at \(-60 \text{ mV}\), the light intensity was reduced to the point where a decrease in the rise time of oEPSCs was clearly distinguished (1 ms duration, 0.2–2.0 mW/mm\(^2\)) (20–80% rise time: before; 1.14 ± 0.22 ms, after; 2.99 ± 0.31 ms, n = 9, p < 0.01, paired t test). Only cells with a constant recording of oEPSCs at a slow rise time (NMDAR-oEPSCs) during baseline were used for the experiments. Series resistance (8–18 M\(\Omega\)) was uncompensated and monitored throughout experiments with a \(-5 \text{ mV}, 50 \text{ ms voltage step}\), and cells that exhibited a significant change in the series resistance more than 20% were excluded from analysis.

For current-clamp recordings, we used an intracellular solution with the following composition (in mM): 136 K-glutamate, 4 KCl, 10 HEPES, 0.2 EGTA, 5 NaCl, 2 MgATP, 0.3 Na\(_3\)GTP, 10 phosphocreatine, pH 7.3 adjusted with KOH (288–294 mOsm). In Fig. 6C, 20 mM K-glutamate was replaced with 20 mM BAPTA. Input resistance was monitored throughout the experiments with a hyperpolarizing current injection (200 ms, 20 pA), and data were excluded if the input resistance changed by more than 30%. Burst-firing-induced LTP (Fig. 1E) was induced in current-clamp mode by theta frequency current injection (10 bursts of 40 ms current injection, which elicited 3–4 APs, at 5 Hz, repeated 5 times every 5 s). For TBS, the stimulation electrode was placed in the middle molecular layer to activate the MPP, and TBS was induced by a series of 10 bursts of 5 stimuli (100 Hz within the burst, 200 ms interburst interval) delivered five times every 10 s.

For field potential recordings, a recording pipette filled with 1 M NaCl was placed in the stratum lucidum of the CA3 region, while a stimulating pipette filled with the ACSF was placed in the DG GC layer. Mossy fiber LTP was induced by a 1 s, 100 Hz stimulation repeated twice at 10 s intervals.

For gramicidin perforated-patch recordings, 200 \(\mu\)g/mL gramicidin (Sigma-Aldrich) and Alexa Fluor 568 (Thermo Fisher Scientific) were included in an intracellular solution (in mM): 64.5 K-glutamate, 57 KCl, 10 HEPES, 0.5 EGTA, 1 CaCl\(_2\), 1 MgCl\(_2\) pH 7.3 adjusted with KOH (290–292 mOsm). Recordings were started when the series resistance fell below 100 M\(\Omega\). Rupture of the patch was confirmed by the presence of Alexa Fluor 568 in the soma.

Experiments for post-tetanic potentiation at parallel fiber (PF)-Purkinje cell (PC) synapses in the cerebellar slices were performed as previously reported (6). Parasagittal cerebellar slices (250 \(\mu\)m) were prepared from 3–4-week-old C57BL/6 mice using
preparation methods similar to those described above. Whole-cell voltage-clamp recordings were made from visually identified PCs ($V_{\text{hold}} = -60$ mV). Patch pipettes (3–5 MΩ) were filled with Cs-based intracellular solution used in the experiments in hippocampal slices. A stimulation pipette was placed in the molecular layer to activate PF inputs, and PF-EPSCs were recorded at 0.5 Hz. Post-tetanic potentiation at PF-PC synapses was induced by 10 pulses delivered at 100 Hz. Throughout the experiments, the CB₁ receptor antagonist AM251 (4 µM) was included in the bath solution to block endocannabinoid-mediated retrograde suppression (6, 7).

Recordings were performed using an EPC10 (HEKA Electronik) or IPA amplifier (Sutter Instruments). Data were filtered at 2.9 kHz and sampled at 20 kHz. Liquid junction potentials were not corrected, unless otherwise stated. Bath solutions contained picrotoxin (100 µM) for recordings of EPSC(P)s, and NBQX (10 µM) and D-AP5 (50 µM) for recordings of IPSCs, unless otherwise stated. In SI Appendix, Fig. S3A, 4 µM AM251 was also included in the bath solution to block depolarization-induced suppression of inhibition (8). All experiments were performed at 28 ± 1°C, except for Fig. 1E, 4H, 6, 7, and SI Appendix, Fig. S7, where experiments were performed at near physiological temperature (31–34°C). Temperature was controlled using a temperature controller (TC-324C, Warner Instruments).

**Pharmacology**

Each reagent of stock solution was dissolved in water or DMSO, depending on the manufacture’s recommendation and stored at −20°C. NBQX, D-AP5, DCG-IV, LY341495, and AM251 were purchased from Tocris Bioscience. AIP, Gö6983, CPA, and H89 were purchased from Cayman Chemical. BoTx and DL-TBOA were purchased from R&D systems. KN-93 and picrotoxin were purchased from Tokyo Chemical Industry. Nifedipine was purchased from Nacalai tesque. NEM was purchased from FUJIFILM Wako Chemicals. BAPTA was purchased from Dojindo Laboratories. Reagents were bath applied following dilution into ACSF from stock solutions just before use. For experiments requiring postsynaptic loading reagents, the LTP induction protocol was applied at least 15–20 min after establishing whole-cell configuration. CPA, H89, Gö6983, and KN-93 were preincubated with slices for at least 1 h and were always included in the bath solution. Control and test conditions were interleaved for all experiments.
**Histology and fluorescence imaging**

For *post hoc* confirmation of the injection site, SuM-containing brain blocks obtained in the preparation of hippocampal slices were kept overnight in 4% PFA and then washed in PBS. Tissues were embedded in 1.5% low-melting-point agarose and sagittally sectioned at 100 µm using a vibratome (DTK-1000N, Dosaka). Fluorescence images were acquired using a fluorescence microscope (BZ-X800, Keyence).

To study the expression of ChR2(H134R)-eYFP, ChR2(H134R)-mCherry, and Venus, AAV-injected mice under deep pentobarbital anesthesia (100 mg/kg of body weight, intraperitoneally) were perfused with 4% PFA. Brains were removed and stored in 4% PFA for 4 h at room temperature, then transferred to PBS and left overnight at 4°C. Coronal brain slices containing the SuM or the hippocampus were sectioned at 100 µm using a vibratome (DTK-1000N, Dosaka). Sections were rinsed twice in PBS and mounted on glass slides with DAPI. For *post hoc* morphological analysis of the recorded neurons, slices were fixed in 4% PFA overnight at 4°C. Slices were then washed with PBS and incubated with streptavidin-conjugated Alexa Fluor 568 (1:500, Thermo Fisher Scientific) in PBS and 0.1% Triton X-100 overnight at room temperature. After washing 5 times with PBS, the slices were mounted on glass slides with DAPI. Fluorescence images were acquired using a confocal microscope (TCS SP8, Leica microsystems) and analyzed using the ImageJ (NIH).

**Data analysis**

The magnitude of LTP was determined by comparing 10 min baseline responses (or 5 min in Fig. 1B, 6, and *SI Appendix*, Fig. S3B) with the last 10 min responses after LTP induction shown in each experiment. The magnitude of TBOA-induced potentiation was determined by comparing 10 min baseline responses with responses 20–30 min after TBOA application. PPR was defined as the ratio of the amplitude of the second EPSC to the amplitude of the first EPSC (100 ms interstimulus interval). PPR was measured 10 min before and 30–40 min (or 0–5 min for Fig. 2D; 20–30 min for *SI Appendix*, Fig. S7C) after LTP induction or TBOA-induced potentiation. The asynchronous events were measured during a 700 ms period beginning 30 ms after light stimulus in order to exclude the initial synchronous synaptic responses. The amplitude and frequency of the asynchronous events were compared before (10 min baseline in the presence of D-AP5) and after (20 min after depol-eLTP induction). Synaptic responses, in which the amplitude of oEPSCs was < 8 pA, were determined to be failures. The failure rate, efficacy (mean EPSC amplitude including failures), and potency (mean EPSC amplitude excluding failures) were compared before (10 min baseline in the presence of D-AP5) with LTP (30 min after LTP induction).
Spike probability was calculated as the number of spikes normalized to the total number of spikes per burst. Post-tetanic potentiation was calculated as the percentage of potentiation in the mean of the 5 consecutive EPSC amplitudes following a burst of PF stimulation relative to the baseline EPSC amplitudes. Averaged representative traces included 10–30 consecutive individual responses.

**Statistics**
Statistical analysis was performed using OriginPro software (OriginLab, USA). The normality of distributions was assessed using the Shapiro–Wilk test. For samples with normal distributions, Student’s unpaired and paired two-tailed t-tests were used to assess between-group and within-group differences, respectively. For samples that were not normally distributed, the non-parametric paired sample Wilcoxon signed rank test and Mann–Whitney U test were used. Differences among two or multiple samples were assessed by using one way or two-way ANOVA, followed by post hoc Tukey’s test if necessary. Kolmogorov–Smirnov test was used for cumulative distributions. Statistical significance was set to p < 0.05 (***, **, and * indicates p < 0.001, p < 0.01 and p < 0.05, respectively). All values are reported as the mean ± s.e.m.

**SI References**
1. Y. Wang et al., Fluorescent labeling of both GABAergic and glycinergic neurons in vesicular GABA transporter (VGAT)-venus transgenic mouse. Neuroscience 164, 1031-1043 (2009).
2. C. Schmidt-Hieber, P. Jonas, J. Bischofberger, Enhanced synaptic plasticity in newly generated granule cells of the adult hippocampus. Nature 429, 184-187 (2004).
3. Y. Hashimotodani, F. Karube, Y. Yanagawa, F. Fujiyama, M. Kano, Supramammillary Nucleus Afferents to the Dentate Gyrus Co-release Glutamate and GABA and Potentiate Granule Cell Output. Cell Rep 25, 2704-2715 e2704 (2018).
4. V. Chevaleyre, S. A. Siegelbaum, Strong CA2 pyramidal neuron synapses define a powerful disynaptic cortico-hippocampal loop. Neuron 66, 560-572 (2010).
5. K. Kohara et al., Cell type-specific genetic and optogenetic tools reveal hippocampal CA2 circuits. Nat Neurosci 17, 269-279 (2014).
6. Y. Hashimotodani et al., Acute inhibition of diacylglycerol lipase blocks endocannabinoid-mediated retrograde signalling: evidence for on-demand biosynthesis of 2-arachidonoylglycerol. J Physiol 591, 4765-4776 (2013).
7. M. Beierlein, D. Fioravante, W. G. Regehr, Differential expression of posttetanic potentiation and retrograde signaling mediate target-dependent short-term synaptic plasticity. Neuron 54, 949-959 (2007).

8. Y. Hashimotodani, T. Ohno-Shosaku, M. Kano, Endocannabinoids and synaptic function in the CNS. Neuroscientist 13, 127-137 (2007).
Fig. S1. Confirmation of AAV injection site in the SuM and projection site of ChR2-eYFP in the hippocampus.

(A-C) Example fluorescence images of confirming the location of the AAV injection site in the SuM from three different mice (top) and corresponding projection site in the hippocampus (bottom). Note that non-fixed hippocampal slices (300 µm thick) used for electrophysiology are shown. Bright field and fluorescence images are overlaid. SuM, supramammillary nucleus; MB, mammillary bodies; IPL, interpeduncular nucleus, lateral subnucleus; Pn, pontine nucleus; VTA, ventral tegmental area; fr, fasciculus retroflexus; mt, mammillothalamic tract; opt, optic tract; f, fornix; SNR, substantia nigra; ml, medial lemniscus.
Fig. S2. The current-voltage relationship of NMDAR-mediated oEPSCs at SuM-GC synapses.
NMDAR-mediated oEPSCs at SuM-GC synapses were recorded in the presence of 10 µM NBQX and 100 µM picrotoxin. 1.3 mM Mg$^{2+}$ was included in the extracellular solution. Representative traces at different membrane potentials (left) and summary plot (right, n = 5) show typical non-linear current-voltage curve for NMDARs. Liquid junction potential of 11 mV was corrected.
Fig. S3. Absence of LTP at IN-GC or SuM-IN synapses following postsynaptic depolarization or the pairing protocol, respectively.

(A) Left, schematic diagram. Stimulation electrodes were placed in the middle molecular layer (MML) or GCL to activate GABAergic axons at two different layers. Both IPSCs were recorded from GCs at −60 mV. Right, the depol-eLTP induction protocol (arrow) had no effects on both IPSCs (MML: 94 ± 8% of baseline, n = 6, p = 0.079, paired t test; GCL: 98 ± 10% of baseline, n = 6, p = 0.85, paired t test). IPSCs were recorded in the presence of CB1 receptor antagonist AM251 (4 µM) to block depolarization-induced suppression of inhibition (DSI). (B) Left, schematic diagram illustrating recording configuration. ChR2(H134R)-mCherry expressing SuM axons were activated by blue light, while whole-cell recording was made from a Venus-positive IN. Right, a pairing protocol (200 light pulses at 2 Hz, paired with 0 mV postsynaptic depolarization, arrow) failed to induce LTP of SuM-IN oEPSCs (93 ± 7% of baseline, n = 5, p = 0.24, paired t test). Data are presented as mean ± SEM.
Fig. S4. Positive control for the validity of the PKA and PKC inhibitors. 
(A) Schematic diagram illustrating extracellular field excitatory postsynaptic potential recording (fEPSP) configuration. A stimulating electrode was placed in the DG GC layer to activate mossy fiber inputs. A recording electrode was placed in the stratum lucidum of CA3 region. (B) Representative traces (left) and summary plot (right) showing that H89 (10 µM) blocked mossy fiber LTP (control: 160 ± 9% of baseline, n = 5; H89: 111 ± 6% of baseline, n = 7; control versus H89: p < 0.001, unpaired t test). Mossy fiber LTP was induced by 100 pulses at 100 Hz repeated twice at 10 s intervals (arrow). DCG-IV (1 µM) was applied at the end of experiments in order to confirm the identity of mossy fiber synaptic transmission. For clarity, first point after LTP induction was removed. (C) Recording configuration in the cerebellar slice. Whole-cell recording was made from Purkinje cell (PC), while parallel fiber (PF) inputs originated from cerebellar granule cell (GC) were electrically stimulated. (D) Representative traces (left) and summary plot (right) showing that post-tetanic potentiation at PF-PC synapses was blocked by the PKC inhibitor Gö6983 (1 µM) (control: 156 ± 11% of baseline, n = 6; Gö6983: 114 ± 8% of baseline, n = 6; control versus Gö6983: p < 0.001, unpaired t test). For induction of post-tetanic potentiation, PFs were stimulated by 10 pulses at 100 Hz at time 0 (arrow). AM251 (4 µM) was included in the bath solution to block endocannabinoid-mediated retrograde suppression. Data are presented as mean ± SEM.
Fig. S5. Recovery from suppression of SuM-GC NMDAR-oEPSCs by D-AP5. Suppression of SuM-GC NMDAR-oEPSCs by bath application of D-AP5 (10 min, 50 µM) was recovered to baseline level 30 min after washout (45-50 min; 94 ± 7% of baseline, n = 4, p = 0.28, paired t test). Data are presented as mean ± SEM.
Fig. S6. Induction of depol-eLTP and measuring $E_{\text{GABA}}$ using gramicidin perforated-patch clamp recordings.

(A) Depol-eLTP was induced by repeated postsynaptic depolarizations (arrow) in the gramicidin perforated-patch clamp configuration (212 ± 53% of baseline, n = 8, p < 0.05, Wilcoxon signed rank test). (B) SuM-GC oIPSCs were recorded in the gramicidin perforated-patch clamp mode in the presence of 10 µM NBQX and 50 µM D-AP5. Representative traces at different membrane potentials are shown on the left. (Right) Summary plot indicates that $E_{\text{GABA}}$ is approximately −75 mV (n = 13). Data are presented as mean ± SEM.
Fig. S7. Inhibition of glutamate uptake increases SuM-GC oEPSCs

(A) Representative SuM-GC oEPSC traces (left) obtained from before (black) and during bath application of glutamate transporter inhibitor TBOA (50 µM, red). Scaled traces are shown on the right. Expanded timescale of 1st EPSC traces (bottom) indicate the prolonged decay time of EPSC by TBOA. (B) Summary plot showing that 30 min bath application of TBOA increases the amplitude of SuM-GC oEPSCs (146 ± 15% of baseline, n = 10, p < 0.01, paired t test), but not electrically-evoked MPP-EPSCs (100 ± 19% of baseline, n = 6, p = 0.622, paired t test). To isolate AMPAR-mediated synaptic transmission, NMDARs, metabotropic glutamate receptors, and GABA_{A}Rs were blocked by D-AP5 (50 µM), LY341495 (100 µM), and picrotoxin (100 µM), respectively. At the end of experiments, 10 µM NBQX was applied to verify that the response was mediated by AMPARs. (C) PPR was not changed following application of TBOA (before: 0.50 ± 0.05; after: 0.49 ± 0.06, n = 10, p = 0.262, Wilcoxon signed rank test), suggesting a postsynaptic origin of this enhancement. (D) Decay time constant (tau) of SuM-GC oEPSCs was increased by TBOA (before: 3.1 ± 0.28 ms; after: 4.2 ± 0.34 ms, n = 10, p < 0.01, paired t test). Data are presented as mean ± SEM. **p < 0.01. n.s., not significant.