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Hypothalamic Glutamate/GABA Co-transmission Modulates Hippocampal Circuits and Supports Long-term Potentiation

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Declaration of interest

The authors declare no competing interests.

AUTHORS CONTRIBUTIONS

M.I.A. and C.C.L. designed the experiments. M.I.A and W.I.A executed the experiments. M.I.A., J.W.W., W.I.A., and C.C.L. analyzed the data. M.I.A. and C.C.L. wrote the paper. C.C.L. acquired the funding.

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Abstract

Subcortical input engages in cortico-hippocampal information processing. Neurons of the hypothalamic supramammillary nucleus (SuM) innervate the dentate gyrus (DG) by co-releasing two contrasting fast neurotransmitters, glutamate and GABA and thereby support spatial navigation and contextual memory. However, the synaptic mechanisms by which SuM neurons regulate the DG activity and synaptic plasticity are not well understood. The DG comprises excitatory granule cells (GCs) as well as inhibitory interneurons (INs). Combining optogenetic, electrophysiological, and pharmacological approaches, we demonstrate that the SuM input differentially regulates the activities of different DG neurons in mice of either sex via distinct synaptic mechanisms. Although SuM activation results in synaptic excitation and inhibition in all postsynaptic cells, the ratio of these two components is variable and cell type-dependent. Specifically, dendrite-targeting INs receive predominantly synaptic excitation, whereas soma-targeting INs and GCs receive primarily synaptic inhibition. Although SuM excitation alone is insufficient to excite GCs, it enhances the GC spiking precision and reduces the latencies in response to excitatory drives. Furthermore, SuM excitation enhances the GC spiking in response to the cortical input, thereby promoting induction of long-term potentiation at cortical-GC synapses. Collectively, these findings provide physiological significance of the co-transmission of glutamate/GABA by SuM neurons in the DG network.

Keywords: Hypothalamus, supramammillary nucleus, co-transmission, dentate gyrus, glutamate, GABA, spike-timing precision, excitability
Significance Statement

The cortical-hippocampal pathways transfer mnemonic information during memory acquisition and retrieval, whereas subcortical input engages in modulation of communication between the cortex and hippocampus. The supramammillary nucleus (SuM) neurons of the hypothalamus innervate the dentate gyrus (DG) by co-releasing glutamate and GABA onto granule cells (GCs) and interneurons and support memories. However, how the SuM input regulates the activity of various DG cell types and thereby contributes to synaptic plasticity remains unexplored. Combining optogenetic and electrophysiological approaches, we demonstrate that the SuM input differentially regulates DG cell dynamics and consequently enhances GC excitability as well as synaptic plasticity at cortical input-GC synapses. Our findings highlight a significant role of glutamate/GABA co-transmission in regulating the input-output dynamics of DG circuits.
Introduction

The cortical-hippocampal pathways transfer mnemonic information during memory acquisition and retrieval and play a central role in spatial navigation, declarative memory, and complex information processing (Amaral et al., 2007; Buzsáki and Moser, 2013; Hainmueller and Bartos, 2020; Henze et al., 2002; Ito et al., 2018; Morris et al., 1982; Nakashiba et al., 2012; Squire, 1992). The synapses present along this pathway have been characterized extensively as substrates for distinct types of memories (Kitamura et al., 2015; Nakazawa et al., 2002; O'Keefe and Dostrovsky, 1971; Remondes and Schuman, 2004). The granule cells (GCs), the principal cells of the dentate gyrus (DG), receive cortical inputs (Buckmaster et al., 1996; Scharfman and Myers, 2012; Zhang et al., 2013) and segregate them into distinct neural codes after integration (Fernández-Ruiz et al., 2021; Yassa and Stark, 2011). In addition, subcortical inputs from different areas of the brain innervate GCs (Leranth and Nitsch, 1994; Nyakas et al., 1987; Unal et al., 2015). However, information regarding the synaptic organization and functions of the subcortical inputs is relatively limited.

The supramammillary nucleus (SuM) in the hypothalamus consist of subsets of neurons that innervate the DG and the CA2/CA3 subfields (Borhegyi and Leranth, 1997; Chen et al., 2020; Kohara et al., 2014; Leranth and Hajsan, 2007; Magloczky et al., 1994; Nitsch and Leranth, 1994; Pan and McNaughton, 2004; Vertes, 2015). SuM-DG connections are known to regulate hippocampal theta oscillations (Ito et al., 2018; Kocsis and Kaminski, 2006; Kocsis and Vertes, 1994; Ruan et al., 2011; Thinschmidt et al., 1995), learning (Aranda et al., 2008; Hernandez-Perez et al., 2015; Ruan et al., 2011; Shahidi et al., 2004), rapid eye movement sleep (Renouard et al., 2015), arousal (Pedersen et al., 2017), and explorative locomotor activities (Ito et al., 2009; Slawinska and Kasicki, 1998;
Moreover, the SuM synchronizes with the DG in the regulation of goal-directed behavior during spatial navigation (Ito et al., 2018; Li et al., 2020). Recently, a subset of SuM neurons was reported to signal contextual information to the DG (Chen et al., 2020).

SuM neurons form synapses with the perisomatic region of the GC and their axonal terminals co-express the vesicular glutamate transporter type II (VGlut2) and vesicular GABA transporter (VGAT) (Boulland et al., 2009; Root et al., 2018; Soussi et al., 2010). Notably, VGlut2 and VGAT are segregated to distinct synaptic vesicles at the SuM terminals in the DG (Boulland et al., 2009; Root et al., 2018). The segregated localization of neurotransmitter vesicles in the same terminals suggests differential co-transmission of glutamate and GABA at the SuM-DG synapses (Dugué et al., 2005; Somogyi, 2006; Vaaga et al., 2014). Consistent with this observation, SuM terminals in the DG simultaneously release both glutamate and GABA to GCs and GABAergic INs (Billwiller et al., 2020; Hashimotodani et al., 2018; Pedersen et al., 2017). Of note, the ratio of the glutamate- and GABA-mediated components recorded in INs varied from 0.34 to 7.7 (Hashimotodani et al., 2018). Given the diverse types of INs present in the DG, glutamate and GABA are likely to be differentially co-transmitted in an IN subtype-specific manner (Booker and Vida, 2018; Hájos et al., 1996; Hosp et al., 2014; Hsu et al., 2016; Liu et al., 2014). Differential recruitment of distinct IN subtypes can powerfully modulate the input and output logic of DG (Lee et al., 2016; Miles et al., 1996). However, whether the SuM input differentially recruits distinct IN subtypes in the DG remains unknown. Moreover, GABA, which is co-transmitted with glutamate by the SuM, is known to exert shunting inhibitory effects on GCs and thereby could bi-directionally control action potential firing in GCs (Chiang et al., 2012; Heigele et al., 2016). Yet it is unclear how the SuM input regulates the input-output dynamics of DG circuits.
Here, combining electrophysiological and optogenetic approaches, we demonstrate that SuM input differentially regulates the activity of DG neurons. Optogenetic activation of SuM input was able to excite dendrite-targeting INs (D-INs), but was not sufficient to activate soma-targeting INs (S-INs) and GCs. Consistent with these observations, GCs and S-INs received predominantly synaptic inhibition, whereas, D-INs received predominantly synaptic excitation. As a consequence, activation of the SuM input enhances the temporal precision of GC firing and shortened spike latencies in D-INs. Moreover, co-activation of the SuM input with the cortical input enhanced the responses of GCs to the cortical input. Finally, repeated co-activation of the SuM and cortical inputs resulted in enhanced long-term potentiation (LTP) at the cortical-GC synapses.
**Materials and Methods**

**Animals**

We used the VGluT2-Cre driver line (Slc17a6^tm2(cre)Lowl/J, stock# 016963), VGAT-Cre driver line (Slc32a1^tm2(cre)Lowl/J, stock# 028862), Gad2-Cre driver line (Gad2^tm2(cre)Zjh/J, stock# 010802) obtained from Jackson Laboratory (Bar Harbor, ME, USA), and wild-type (WT) mice with C57BL/6J genetic background obtained from National Laboratory Animal Center (Taipei, Taiwan). Both male and female mice (3-5 months old) were used for the electrophysiological experiments. The mice were housed in a room with a reverse 12-h light-12-h-dark cycle and were provided with food and water *ad libitum*. The protocols and procedures for the animal experiments were in accordance with the national and institutional guidelines and were approved by the Animal Care and Use Committee of National Yang Ming Chiao Tung University.

**Viruses**

For the optogenetic experiments, we virally expressed channelrhodopsin (ChR2)-eYFP on SuM neurons by injecting an adeno-associated virus (AAV) serotype 5-CaMKIIα-ChR2(H134R)-eYFP (4.1×10^{12} vector genomes/mL, University of North Carolina, Chapel Hill, NC, USA) into the SuM of WT mice. To target glutamatergic and GABAergic neurons in the SuM selectively, an AAV5 vector carrying a Cre-inducible ChR2-eYFP transgene (AAV5-EF1α-DIO-hChR2-(H134R)-eYFP) (4.3×10^{12} vector genomes/mL, University of North Carolina, Chapel Hill, NC, USA) was injected into the SuM of VGluT2-Cre, VGAT-Cre and Gad2-Cre mice.

**Stereotaxic injection**

For the retrograde tracer and virus injections, the mice were anesthetized with 4% isoflurane (v/v; Halocarbon Laboratories, North Augusta, SC, USA) in a 100% oxygen-containing induction chamber. The scalp was shaved and the mice were transferred to a...
stereotaxic frame (IVM-3000; Scientifica, Uckfield, UK) for the surgery. The mouth and nose of each mouse were covered using an anesthetizing mask that was supplied with approximately 1.5% isoflurane and had an airflow rate of 4 mL/min. To maintain the body temperature of the mice at 34–36°C, a biological temperature controller pad (Physitemp Instruments, New Jersey, USA, or TMP-5b, Supertech Instruments, Budapest, Hungary) remained placed under the body of each mouse throughout the surgical procedure. The head was fixed using two ear bars; 75% ethanol was applied to the scalp to sterilize the surgical area, and an ophthalmic gel was applied to the eyes to avoid dryness. An analgesic (ketorolac, 6 mg/kg) was administered intraperitoneally. For the delivery of the tracer, unilateral or bilateral craniotomy was performed at the antero-posterior (AP) and medio-lateral (ML) coordinates of the dorsal DG (AP: -1.80 mm, ML: ± 1.30 mm). Then the tracer was delivered into the DG at the dorso-ventral (DV) coordinate (DV: -2.20 and -2.0 mm). To target the SuM neurons, unilateral or bilateral craniotomy was performed over the SuM (AP: -2.85 mm, ML: ± 0.15 mm). Then viral vectors were delivered into the SuM at DV, -4.86 mm. The viral vectors (0.2–0.4 μL) and red retrobeads (0.2 μL) (LumaFlour, North California, USA) were delivered to the SuM and DG, respectively, using a 10-μL NanoFil syringe (World Precision Instruments, Sarasota, FL, USA) and a 34-G beveled metal needle. The injection volume (0.2–0.4 μL) and flow rate (0.1 μL/min) were controlled using a nanopump controller (KD Scientific, Holliston, MA, USA). Subsequently, the needle was raised 0.1 mm above the site of injection for an additional 10 min to minimize the upward flow of the viral solution. Finally, the needle was gradually withdrawn. After the injection was performed, the incision was sutured, and the mice were transferred to the cage for recovery.

Preparation of brain slices
Acute brain slices containing the hippocampal and SuM sections were prepared one week after the retrograde tracer injection or at least three weeks after the viral injection. Transverse brain slices were used for whole-cell patch-clamp recording of the DG neurons, while coronal brain slices were used for recording of retrobeads positive SuM neurons. The mice were anesthetized using isoflurane and decapitated rapidly. The brains were quickly removed and transferred to an ice-cold oxygenated (95% O₂ and 5% CO₂) sucrose solution containing (in mM): 87 NaCl, 25 NaHCO₃, 1.25 NaH₂PO₄, 2.5 KCl, 10 glucose, 75 sucrose, 0.5 CaCl₂, and 7 MgCl₂. Next, 300 μm thick slices were cut using a vibratome (DTK-1000; Dosaka, Kyoto, Japan). After sectioning, the slices were recovered at 34°C for 25 min in a holding chamber filled with an oxygenated sucrose solution, then transferred to room temperature (25 ± 2°C) for further experiments.

**Electrophysiology and optical stimulation**

For the recordings, individual slices were transferred to a submerged chamber and were continuously perfused with oxygenated artificial cerebrospinal fluid (ACSF) containing the following (in mM): 125 NaCl, 25 NaHCO₃, 1.25 NaH₂PO₄, 2.5 KCl, 25 glucose, 2 CaCl₂, and 1 MgCl₂. The ChR2-eYFP expression pattern was confirmed using fluorescence and the neurons in the DG were selected visually for recording under an infrared differential interference contrast microscope (IR-DIC, BX51WI, Olympus). The axonal terminals that expressed ChR2 were stimulated with 470-nm light transmitted through the objective from an LED source (LED4D162, driven by DC4104, Thorlabs, Newton, NJ, USA).

Whole-cell patch-clamp recordings were performed using a Multiclamp 700B amplifier (Molecular Devices, Sunnyvale, CA, USA). The recording electrode pipettes (4–7 MΩ) pulled from borosilicate glass tubing (outer diameter, 1.5 mm; inner diameter, 0.86 mm; Harvard Apparatus) were filled with a high-Cl⁻ internal solution, containing the following (in mM): 15 K-gluconate, 140 KCl, 0.1 EGTA, 2 MgCl₂, 4 Na₂ATP, 10 HEPES,
0.5 Na$_3$GTP, and 0.4% biocytin (w/v, Life Technologies, Grand Island, NY, USA). In certain set experiments for the determination of spike-timing precision and spike phase, a low Cl$^{-}$ internal solution containing (in mM): 136.8 K-gluconate, 7.2 KCl, 0.2 EGTA, 4 MgATP, 10 HEPES, 0.5 Na$_3$GTP, 7 Na$_2$-phosphoreatine (pH 7.3 with KOH) and 0.4% biocytin was used. The pipette capacitance was compensated in the cell-attached mode. To measure the excitatory postsynaptic current (EPSC) and the inhibitory postsynaptic current (IPSC), whole-cell recording was performed using a high Cl$^{-}$ internal solution (E$_{GABA}$ = ~0 mV, E$_{AMPA}$ = ~0 mV), the EPSC and IPSC were isolated using a pharmacological approach. Bath application of SR95531 (1 μM) and CGP55845 (1 μM) were used to block GABA$_A$ and GABA$_B$ receptors, respectively, while an ionotropic glutamate receptor blocker, kynurenic acid (Kyn, 2 mM) was used to block ionotropic glutamatergic transmission. The GABAergic component (IPSC) traces were obtained by digital subtraction of traces recorded after bath application of SR, CGP from the baseline traces recorded in the presence of ACSF. The glutamatergic component (EPSC) traces were obtained by digital subtraction of traces recorded in the presence of SR, CGP and Kyn from the traces recorded in the presence of SR and CGP.

Cell-attached was performed with patch pipettes filled with a high Cl$^{-}$ internal solution before whole-cell recording of current spikes in GCs and INs. A 5-Hz, 5 ms light pulse was applied with a 15-s inter-sweep interval and 6 sweeps were recorded. The spike probability was determined as the percentage of spikes among 6 sweeps. In the dual recording experiments, the distance between the recorded pair was less than 200 μm. Although the serial resistance was not compensated, it was monitored continuously during the recording process. The recordings with the serial resistance < 25 MΩ were analyzed. Fast-spiking phenotype of hippocampal INs or putative S-INs recorded at room temperature (21-24°C) were defined by their maximal firing rate > 65 Hz and coefficient of
variation (CV) of < 0.2 in response to 1-s depolarizing current injection (Lien and Jonas, 2003). The CV was determined from the spike train with the maximal firing rate. For local field potential recordings, a monopolar electrode (tip diameter; ~10 μm) filled with ACSF was placed in the subiculum to stimulate the perforant path (PP) fibers. Trains of current pulses (10–500 μA, 0.1 ms) were applied every 15 s using a stimulus isolator (Isoflex, A.M.P.I., Jerusalem, Israel). The recording electrode (tip diameter, ~5 μm) filled with ACSF was placed in the granule cell layer (GCL) to monitor the population spike (pSpike) in response to PP stimulation. Further experiments were performed at stimulus intensities that evoked 30–50% of the maximum pSpike amplitude and paired with the 10-ms light pulse for activation of the SuM input.

For the spike-timing precision experiments, sinusoidal waveforms were created and customized using Clampfit 10.3 (Molecular Devices). To test the ability of the SuM input to enhance spike-timing precision and phase, theta frequency (5-Hz trains of 5 pulses) sinusoidal current pulses were delivered into the GCs and were paired with 5-Hz square photostimulation of the SuM input. The 5-ms photostimulation was delivered during the ascending phase (31°–39°) of the sinusoidal waveform. The current injected (peak to trough, 50–150 pA) was set to evoke a single action potential (AP) close to the peak of the sinusoidal waveform while the membrane potential of the GCs was held at approximately -80 mV. Twenty sweeps were recorded at 15-s interval and superimposed to observe the precision of AP generation. To determine the spike jitter and phase, the time point for the peak in each spike was converted to phase (angle) using the customized Python codes. The mean and the standard deviation represented spike phase (latency) and spike jitter, respectively. All cells used for spike-timing precision experiments reliably generated excitatory postsynaptic potential (EPSP) in response to 5-Hz photostimulation of the SuM input. The signals were recorded using Multiclamp 700B amplifiers (Molecular Devices),
filtered at 4 kHz and sampled at 10 kHz using a digitizer (Digidata 1440A, Molecular Devices), which was controlled using pCLAMP version 10.3 (Molecular Devices).

**Post-hoc recovery and reconstruction of recorded neurons**

To identify the recorded neurons (filled with 0.4% biocytin), brain slices were fixed overnight with 4% paraformaldehyde (w/v) in phosphate-buffered saline (PBS). After rinsing with PBS 3 times, 0.3% Triton X-100 (v/v; USB Co., Cleveland, OH, USA) in PBS (PBST) was added for 30 min. Then, blocked with 0.3% PBST and 10% normal goat serum (NGS, S-1000, Vector Laboratories, Burlingame, CA, USA) for 2 h. Slices were incubated with streptavidin-conjugated Alexa Fluor 594 or 555 or 488 (1:400; Life Technologies) in 0.3% PBST and 5% NGS at 4°C overnight or 2 h at room temperature. After rinsing 6 times with PBS, slices were mounted onto slides with mounting medium Vectashield with 4′,6-diamidino-2-phenylindole (DAPI, H-1200, Vector Laboratories, Burlingame, CA, USA). Confocal image stacks were reconstructed with Neuromantic 1.6.5 software (developed by Darren Myatt, University of Reading, Reading, Berkshire, UK).

**Immunohistochemistry**

WT mice (3 months old) with AAV5-CaMKIIα-ChR2-eYFP injected into the SuM were deeply anesthetized using isoflurane and perfused transcardially with 20 mL of ice-cold PBS, followed by 50 mL of 4% PFA. The fixed brain specimens were excised and post-fixed in 4% PFA for an additional 6 h or overnight. Next, dehydration was performed by incubation in 15% sucrose for 4 h, followed by 30% sucrose in PBS for 2 h. The brain specimens were sectioned coronally into 50-μm slices using a microtome (SM2010R, Leica, Wetzlar, Germany). The brain slices were rinsed with PBS three times and blocked by treating with 0.3% PBST and 5% NGS for 2 h. The slices were then incubated in a cocktail of rabbit anti-GFP antibody (1:1000, Abcam, ab290), rabbit anti-VGluT2 antibody
Next, the slices were rinsed three times with PBS and incubated in cocktails of fluorescent secondary antibodies, Alexa Fluor 488 anti-rabbit, Alexa Fluor 594 anti-rabbit, and Alexa Fluor 647 anti-mouse at room temperature for 2 h or overnight at 4°C. The procedures were performed under continuous shaking conditions. After rinsing six times with PBS, the sections were mounted using the mounting medium Vectashield with DAPI. Fluorescent images were taken using a confocal microscope (Leica SP5 module, Leica Microsystems, Germany) or (LSM 700, Zeiss, Germany) using 20×, 40× or 63× objectives and analyzed using ImageJ (NIH, USA, 1.52t). Single plane coronal sections with bead expression were imaged using a Research High-Class Stereo Microscope System (SZX16, Olympus, Tokyo, Japan). For colocalization analysis of ChR2-eYFP expressing boutons with VGluT2 and VGAT, boutons along ChR2-eYFP expressing axons were identified in z-stack images and examined for colocalization and were counted using cell counter plugin in Fiji (a distribution of ImageJ software, NIH, USA, 1.53c) (Billwiller et al., 2020).

**Data analysis and statistics**

Data were analyzed using Clampfit 10.3 (Molecular Devices), Prism 6.0 (GraphPad Software, La Jolla, CA, USA), or customized Python codes. The synaptic latency was determined as the time elapsed from the light onset to the onset of the synaptic response (Hsu et al., 2016). The onset of the synaptic response was determined by the intersection of a line through the 20% and 80% points of the rising phase of the EPSC or IPSC and the baseline. To calibrate evoked IPSCs during successive 5-Hz photostimulation, the EPSC obtained after bath application of SR95531 (1 μM) and CGP55845 (1 μM) was digitally subtracted from the mixed postsynaptic current (baseline). To calculate the conductance, the EPSC and the IPSC amplitude were divided by their respective driving forces.
input resistance was determined by the ratio of a steady-state (the last 100 ms of a 1-s pulse) voltage response versus the injected 1-s hyperpolarizing (10 pA) current pulse (Liu et al., 2014). The magnitude of LTP was calculated 30-40 min after LTP induction. Data are presented as mean ± standard error of mean (SEM). Error bars in figures also show SEMs. Statistical significance was tested using the unpaired t test, Mann–Whitney test, Wilcoxon signed-rank test or two-way repeated-measures ANOVA followed by Bonferroni’s post-hoc tests. Significance levels were set at p < 0.05 (*), p < 0.01 (**), p < 0.001 (***) and p < 0.0001(****) for the statistical comparisons.
Results

Anatomical and physiological features of DG-projecting SuM neurons

To identify and characterize the morpho-physiological properties of DG-projecting SuM neurons, a retrograde tracer (red retrobeads) was injected into the bilateral DG in the hippocampus (Figure 1A, left) of three mice. The injection sites were confirmed by post hoc serial coronal sections (Figure 1A, middle). The beads were restricted to the granule cell layer (GCL) and hilus of the DG (Figure 1A, right top). One week after the injection, the retrogradely labeled DG-projecting neurons were detected primarily in the lateral subdivision of the SuM (SuML) above the mammillothalamic tract (mt) (Figure 1A, right bottom). Only few labeled cells were detected in the medial subdivision of the SuM (SuMM) (Figure 1A, right bottom) as reported previously (Soussi et al., 2010). Notably, in mice injected unilaterally in the right DG (Figure 1B), the labeled DG-projecting SuM cells were mostly detected ipsilateral to the injection side (Figure 1C; Figure 1D, data from 12 slices, 3 mice). Next, we performed whole-cell recordings from labeled DG-projecting SuM neurons located in the SuML in brain slices prepared from both bilateral and unilateral DG injected mice (Figure 1E). These cells had large cell bodies (≥ 20 μm in diameter; Figure 1E), with a resting membrane potential of -58.0 ± 1.7 mV (n = 11 cells from 5 mice) and an input resistance of 508.3 ± 69.4 MΩ (n = 11 cells from 5 mice). They exhibited a bursting firing pattern (at holding potential of -70 mV) in response to small current injection (10–30 pA) and displayed an accommodating firing pattern in response to increased depolarizing current (Figure 1F; n = 11 cells from 5 mice). The biocytin-filled SuM cells exhibited axonal projection extending toward the dorsal brain areas with dendrites located within the mammillary region (Figure 1G; n = 5 cells from 4 mice).
Next, we used an optogenetic approach to investigate the function of SuM projections. A CaMKIIα-ChR2-eYFP virus was injected into the SuM of wild-type (WT) mice (Figure 1H, top). The SuM neuron projections were observed to form a dense pattern in the supragranular layer of the GCL and CA2 pyramidal layer (Figure 1H, bottom, from three mice). To confirm that the ChR2-expressing SuM neurons respond to light stimulation, we made whole-cell recording from these neurons (Figure 1I). When the recorded neurons were illuminated with blue light pulses (470 nm, 5 ms at 5 Hz), they generated spikes in current clamp at -70 mV (Figure 1I, traces; n = 7 cells, 5 mice).

Similarly, a light-evoked ChR2-mediated inward current was recorded in voltage clamp in the presence of an ionotropic glutamate receptor antagonist, kynurenic acid (Kyn, 2 mM) (Figure 1I, traces). Consistent with previous studies (Boulland et al., 2009; Hashimotodani et al., 2018; Soussi et al., 2010; Root et al., 2018), the ChR2-eYFP-expressing axon terminals in the DG (Figure 1J) co-expressed VGluT2 and VGAT (Figure 1K). A total of 1381 putative boutons (from 9 slices, 2 mice) were identified along the ChR2-eYFP-expressing axons. Overall, 92 ± 1.4% (85-98%) of the boutons expressed VGluT2, 88 ± 2.3% (82-97%) expressed VGAT while 84 ± 2.3% (78-94%) expressed both VGluT2 and VGAT, similar to previous reports (Billwiller et al., 2020; Root et al., 2018; Soussi et al., 2010).

**SuM input preferentially excites dendrite-targeting INs**

Next, we examined SuM-DG synaptic transmission by recording field excitatory postsynaptic potentials (fEPSPs) along the somatodendritic axis of GCs (Figure 2A, top). The fEPSPs exhibited downward at the GCL (-0.10 ± 0.01 mV; n = 7) and inner molecular layer, IML (-0.06 ± 0.01 mV; n = 7). The polarity of fEPSP reversed at the middle molecular layer, MML (0.03 ± 0.00 mV; n = 7) and exhibited upward at the outer molecular layer,
OML (0.03 ± 0.00 mV; n = 7). This was consistent with the observation that SuM axons mainly innervated the somatic and proximal dendritic regions of GCs (Hashimotodani et al., 2018). Then, we tested whether activation of SuM terminals alone was sufficient to excite any DG neurons. To this end, we injected a CaMKIIα-ChR2-eYFP virus into the SuM of WT mice or EF1α-DIO-ChR2-eYFP virus into VGluT2-Cre mice. Next, cell-attached recordings were performed from various types of DG neurons such as GCs, soma-targeting INs (S-INs), and dendrite-targeting INs (D-INs) (Figure 2A, bottom) and followed by biocytin-filled whole-cell recordings for post hoc morphologically identification (Hsu et al., 2016; Lee et al., 2016; Liu et al., 2014). Dentate GCs receive coherent theta (4–10 Hz)-band EPSCs in vivo (Pernía-Andrade and Jonas 2014) and the SuM synchronizes with the DG (Li et al., 2020). Thus, we investigated the response of DG cells to SuM activation at a physiologically relevant frequency (e.g., 5 Hz). Upon photostimulation of SuM axons (5 Hz, 5 ms pulses), no spikes were evoked in all recorded GCs (Figure 2B; 21/21 cells) and S-INs (Figure 2C; 5/5 cells). In contrast, the majority of D-INs reliably generated spikes in response to SuM terminal activation (Figure 2D; 22/27 cells). Several morphological subtypes of D-INs have been well characterized (Freund and Buzsáki, 1996; Hsu et al., 2016). According to their soma locations and the input layers where their axons innervate, there are at least four distinct subtypes, including the total molecular layer cells (TML cells), hilar perforant path-associated cells (HIPP cells), molecular layer perforant path-associated cells (MOPP cells), and hilar commissural-associational pathway-related cells (HICAP cells) (Figure 2E). Based on the results of morphological reconstructions, the spike probability of each subtype was plotted against the stimulus number (Figure 2F). The five nonresponsive D-INs, including two HICAP, two HIPP and one MOPP were not included in the plots here. Collectively, the SuM input alone was sufficient to activate most D-INs, but not GCs and S-INs.
Differential glutamate/GABA co-transmission is target cell-specific

Synaptic excitation and inhibition are critical for neuronal excitability and information processing in neural circuits (Bhatia et al., 2019; Iascone et al., 2020; Liu et al., 2004; Yizhar et al., 2011). SuM afferents are known to co-release glutamate and GABA onto both GCs and GABAergic INs (Hashimotodani et al., 2018; Li et al., 2020; Pedersen et al., 2017). Given that the SuM input preferentially excite D-INs, we next investigated whether synapse-specific excitation and inhibition correlate with differential recruitment of DG cells.

To address this question, ChR2-eYFP was virally expressed in SuM neurons of WT or VGluT2-Cre mice (Figure 3A, top) and recordings were made from transverse slice sections of the DG (Figure 3A, bottom). The expression of ChR2-eYFP in the GCL was confirmed before recordings (Figures 3B, 3C and 3D, top). To determine the synaptic property at the SuM-GC synapse, we performed whole-cell recordings from GCs, which exhibited regular spiking, at -75 mV ([Cl]$_i$ = 140 mM; $E_{GABA} = \sim 0$ mV as determined experimentally, Figure 3B, bottom) in brain slices. Photostimulation of the SuM terminals (470 nm, 5 ms at 5 Hz) in the DG evoked inward currents in all recorded GCs (30 of 30 cells; 12 mice). The mean peak amplitude was 84.0 ± 7.0 pA (n = 30) at -75 mV. The mean response was largely reduced by co-application of a GABA$_A$ receptor blocker, SR95531 (1 μM) and a GABA$_B$ receptor blocker, CGP55845 (1 μM) to 22.6 ± 2.4 pA and finally almost abolished by Kyn (2 mM) (Figure 3B, traces). The pharmacologically isolated components, SR and CGP-sensitive component (hereafter called “IPSC”) and Kyn-sensitive component (hereafter called “EPSC”) were GABAergic and glutamatergic, respectively (Figure 3B, red trace, EPSC and blue trace, IPSC). The GABAergic component was slower (20 to 80% rise time, 2.79 ± 0.37 ms; n = 30; decay time constant, 30.67 ± 1.85 ms; n = 30) relative to the glutamatergic component (20 to 80% rise time, 1.10 ± 0.07 ms; n = 30; decay time constant, 6.87 ± 0.47 ms; n = 30). Nevertheless, both
EPSC and IPSC components exhibited similar synaptic latencies in response to 5 ms photostimulation of SuM terminals (Figure 3E, EPSC, 2.60 ± 0.10 ms; IPSC, 2.56 ± 0.10 ms; n = 30; p = 0.875, U = 439.0; Mann-Whitney test), supporting the idea of glutamate and GABA co-transmission at the SuM-GC synapse. The EPSC and IPSC evoked by SuM terminal activation exhibited strong depression of the amplitude (Figure 3B, bottom traces). Notably, analysis of the first peak excitatory and inhibitory conductances (hereafter called EPSG and IPSG) revealed that inhibitory transmission dominated at the SuM-GC synapse (Figure 3F, GCs, EPSG, 0.30 ± 0.03 nS; IPSG, 0.91 ± 0.08 nS; n = 30; p < 0.0001; U = 67.0; Mann-Whitney test). Moreover, the scatter plot of individual relationship between EPSG and IPSG obtained from each cell showed a bias towards IPSG (Figure 3G, gray circles) and the slope of the linear regression line (gray line) was less than 1. Taken together, GABAergic transmission was predominant at the SuM to GC synapse.

Next, we investigated the synaptic property of different IN subtypes (Figures 3C and D). Photostimulation of the SuM terminals evoked variable inward currents (Figures 3C and D, black traces) in different IN subtypes. Similar to GCs, the evoked postsynaptic current recorded from putative S-INs, which exhibited fast-spiking firing pattern (see Materials and Methods). In our recording, S-INs exhibited maximum firing rate of 74.0 ± 4.9 Hz (n = 6 cells; 5 mice), was largely blocked by bath application of SR95531 (1 μM) and CGP55845 (1 μM) (Figure 3C, bottom traces). The remaining small excitatory component was blocked by Kyn (2 mM). Overall, 3 of 6 fast-spiking INs were morphologically confirmed as S-INs. The pharmacologically isolated EPSC and IPSC in S-INs have similar synaptic latencies (Figure 3E, S-INs, EPSC, 2.78 ± 0.20 ms; IPSC, 3.07 ± 0.23 ms; n = 6; p = 0.571, U = 14.0; Mann-Whitney test). The 20 to 80% rise time of the IPSC and EPSC was 1.73 ± 0.31 ms and 1.19 ± 0.08 ms (n = 6), respectively, while the decay time constant of IPSC and EPSC was 17.40 ± 1.53 ms and 7.94 ± 0.55 ms (n = 6).
respectively. Like SuM-GC synapses, analysis of EPSG<sub>1</sub> and IPSG<sub>1</sub> showed that inhibitory conductance dominated at the SuM-S-IN synapses (Figure 3F, S-INs, EPSG<sub>1</sub>, 0.58 ± 0.08 nS; IPSG<sub>1</sub>, 2.14 ± 0.67 nS; n = 6; p < 0.05; U = 4.0; Mann-Whitney test). However, the IPSGs at SuM-S-IN synapses were larger than that at SuM-GC synapses (S-INs, IPSG<sub>1</sub>, 2.14 ± 0.67 nS; GCs, IPSG<sub>1</sub>, 0.91 ± 0.08 nS, p < 0.01, unpaired t test). Furthermore, the plot of EPSG<sub>1</sub> versus IPSG<sub>1</sub> showed a bias towards the IPSG<sub>1</sub>, confirming the dominance of inhibitory conductance at the SuM-S-IN synapses (Figure 3G, orange regression line).

Intriguingly, unlike GCs and S-INs, the co-application of GABA<sub>A</sub> receptor blockers SR95531 (1 μM) and CGP55845 (1 μM) slightly reduced the postsynaptic current recorded in most D-INs (Figure 3D, bottom). However, further bath application of Kyn completely blocked the remaining large current, indicating a dominant excitatory transmission at the SuM-D-IN synapses (Figure 3D). The pharmacologically isolated EPSC and IPSC (Figure 2D; EPSC, red trace and IPSC, blue trace) exhibited similar synaptic latencies (Figure 3E, D-INs, EPSC<sub>1</sub>, 2.67 ± 0.09 ms; IPSC<sub>1</sub>, 2.73 ± 0.10 ms; n = 22; p = 0.663, U = 223.0; Mann-Whitney test). The IPSC kinetics was slower (20 to 80% rise time, 2.53 ± 0.23 ms; n = 25; decay time constant, 19.64 ± 2.40 ms; n = 25) relative to the EPSC kinetics (20 to 80% rise time, 1.25 ± 0.11 ms; n = 25; decay time constant, 6.38 ± 0.61 ms; n = 25). Contrary to the SuM-GC and SuM-S-IN synapses, analysis of EPSG<sub>1</sub> and IPSG<sub>1</sub> showed that excitation dominated the SuM-D-IN synapses (Figure 3F, D-INs, EPSG<sub>1</sub>, 0.99 ± 0.08 nS; IPSG<sub>1</sub>, 0.48 ± 0.08 nS; n = 22; p < 0.0001; U = 63; Mann-Whitney test). The plot of EPSG versus IPSG recorded from each cell revealed a clear shift towards excitatory conductance (Figure 3G, violet circles) and the slope was greater than 1 (Figure 3G). In another set of experiments of VGluT2-Cre mouse virally injected with EF1α-DIO-ChR2-eYFP (Figure 4A), the monosynaptic co-transmission of the glutamate and GABA was also pharmacologically verified by adding tetrodotoxin (TTX), a
voltage-dependent sodium channel blocker, and 4-aminopyridine (4-AP), a voltage-dependent potassium channel blocker (Figures 4B, GC and 4E, D-IN). The light-evoked postsynaptic current was completely abolished by bath application of TTX (1 μM) and was reversed by subsequent addition of 4-AP (1 mM; in the presence of TTX). Consistent with a previous report (Hsu et al., 2016), synaptic latencies were significantly increased by 4-AP (Figure 4C; SuM-GC; synaptic latency, baseline, 2.24 ± 0.11 ms; TTX & 4-AP, 4.01 ± 0.28 ms; n = 9 cells; 5 mice; Figure 4F, SuM-D-IN; synaptic latency, baseline, 2.67 ± 0.21 ms; TTX & 4-AP, 3.66 ± 0.17 ms; n = 6 cells; 4 mice). Analysis of the EPSG and IPSG further confirmed that GABAergic transmission dominated at the SuM-GC synapse (Figure 4D), while glutamatergic transmission was predominant at the SuM-D-IN synapse (Figure 4G). Moreover, the scatter plot of all EPSGs and IPSGs obtained from individual cells revealed a slope of 0.14 at the SuM-GC synapse and a slope of 1.40 at the SuM-D-IN synapses (Figure 4H). Similar results were obtained from GCs recorded in VGAT-Cre and Gad2-Cre transgenic mice (Figure 4I-L). In addition to GCs and INs, we also checked the functional connectivity between the SuM input and mossy cells (MCs), which are excitatory neurons located in the hilus and featured by prominent thorny excrescences at their proximal dendrites (Figure 5A). We performed sequential whole cell recordings from GCs and MCs (Figure 5A). Consistent with a recent report that MCs rarely receive synaptic input from SuM (Hashimotodani et al., 2018), only 1 out of 5 MCs (4 mice) recorded received the discernible response to photostimulation of the SuM input (Figure 5D and E) and the current was small (-42 pA; Figure 5E). The summary plot of first EPSG and IPSG obtained from different cell types in the DG are shown in Figures 5C and 5D.

Finally, to exclude the possibility that the distinct synaptic properties observed here was due to variable viral expression from slices to slices, we performed another set of experiments in WT mice (Figure 6A), where simultaneous dual recordings of GCs and D-
INs were obtained from the same slices (Figure 6B). We found that photostimulation of SuM input (5 ms, 470 nm, 5 Hz light pulses) in the DG evoked inward currents in both GCs and INs (Figure 6C, 6 of 7 pairs recorded, black traces). Co-application of SR95531 (1 μM) and CGP55845 (1 μM) blocked approximately 70.5 ± 5.0% of current in GCs, only about 25.5 ± 5.5% was blocked in D-INs and Kyn (2 mM) completely blocked the remaining current in both GCs and D-INs (Figure 6C). The synaptic strength was stronger at the SuM-D-INs synapses compared to that at the SuM-GC synapses (Figure 6D). Consistent with this, analysis of the peak excitatory and inhibitory conductances (EPSP$_1$ and IPSP$_1$) in some cells revealed that inhibitory transmission dominated at the SuM-GC synapses (Figure 6E, left, EPSP$_1$; 0.22 ± 0.05 nS, IPSP$_1$; 0.52 ± 0.10 nS; n = 5 cells; 4 mice; p < 0.05; U = 2.0; Mann-Whitney test), while excitatory transmission dominated at the SuM-D-IN synapses (Figure 6E, right, EPSP$_1$; 1.24 ± 0.26 nS, IPSP$_1$; 0.40 ± 0.09 nS; n = 5 cells; 4 mice; p < 0.01; U = 0.0; Mann-Whitney test). Taken together, these results demonstrated that the ratio of excitatory and inhibitory components at SuM-DG synapses depends on the subtypes of target cells.

**SuM input shortens spike latency and enhances spike-timing precision**

Cortical principal neurons fire with large variability in response to identical stimuli *in vivo* (Carandini, 2004; Fricker and Miles, 2001; Shadlen and Newsome, 1998). Well-timed inhibition from GABAergic transmission is known to promote precise spike timing, that is essential for hippocampal network oscillation and is thought to be critical for several cognitive functions (Bacci and Huguenard, 2006; Hou et al., 2016; Woodruff and Sah, 2007). Here, we explored how SuM-driven synaptic excitatory and inhibitory conductances regulate spike generation in GCs and D-INs using the low chloride internal solution [Cl$^-$]$_i$ = 7.2 mM, which is close to the physiological intracellular chloride concentration (Chiang et al., 2012). To simulate *in vivo* membrane oscillations, GCs and D-INs were driven by
injecting sinusoidal current steps at low theta (5 Hz) frequencies (Figure 7). Under this condition, photostimulation of the SuM input at the ascending phase of each theta cycle slightly increased spike numbers in GCs (Figures 7A and B; see Materials and Methods). Given that D-INs received predominantly synaptic excitation upon SuM activation, we next examined the modulatory effect of SuM activation on spike generation in D-INs in response to the same oscillatory input. Compared with the light-off epoch, photostimulation of the SuM input remarkably increased spike numbers in D-INs in response to sinusoidal current injections (Figures 7C and D). Next, we examined the latency and spike jitter in GCs and D-INs by injecting a constant suprathreshold sinusoidal current, which was near enough to generate single spikes near the peak of each theta cycle (GCs, Figure 7E; D-INs, Figure 7H). Superimposition of spike trains from GCs (Figure 7E) showed that SuM stimulation shortened the spike latencies and decreased spike jitters (Figure 7E, traces). Both reduction in spike latencies and jitters were only significant in 1st spike (Figures 7F and G), which could be explained by strong synaptic depression at the SuM to GC synapses. Notably, superimposition of spike trains from D-INs showed that pairing the SuM input with the suprathreshold sinusoidal stimulation (baseline-to-peak current amplitude of 80 pA) greatly reduced spike latencies (Figure 7I). In great contrast to GCs, photostimulation of the SuM input did not have a significant effect on spike jitters in D-INs (Figure 7J). This result was consistent with our observation of high synaptic excitation and low synaptic inhibition at the SuM-D-IN synapses. Taken together, activation of SuM input differentially regulates spike generation in GCs and D-INs.

**SuM input enhances GC excitability, thereby supporting long-term potentiation**

Subcortical inputs modulate GC responses to cortical inputs *in vivo* (Nakanishi et al., 2001; Li et al., 2020). In the DG circuits, the equilibrium potential of GABAergic conductance (E\textsubscript{GABA}) is approximately -72 mV (Chiang et al., 2012), which is more depolarized than the
resting potential of GCs (ranging from -80 to -90 mV). Thus, GABA, which is co-transmitted with glutamate by the SuM, could exert either the ‘shunting inhibitory’ or ‘depolarizing (or excitatory)’ effect on GCs. Our previous studies (Chiang et al., 2002; Hsu et al., 2016) report that GABA could promote action potential generation in GCs. Next, we investigated the functional relevance of glutamate/GABA co-transmission on GC responses to the excitatory PP input. We performed local field potential recordings in the GCL in response to photostimulation of the SuM input and/or electrical stimulation of the PP input (Figure 8A). The evoked response consisted of the fEPSP and population spike (pSpike), a proxy of synaptic strength and GC activity, respectively. Photostimulation of the SuM input evoked the fEPSP, but did not generate the pSpike (Figure 8B, black trace), whereas electrical stimulation of the PP generated a compound response, which consisted of the fEPSP followed by the pSpike (Figure 8B, gray area trace). Notably, paired activation of the PP and SuM inputs significantly increased the pSpike area (Figure 8B, blue area trace), indicating an increase in GC spike numbers. The summated trace obtained by digital summation of SuM-fEPSP and PP-response was shown in the red trace (Figure 8B, arithmetic sum). Finally, we overlaid all traces and revealed that the SuM-fEPSP emerged before the onset of pSpikes (Figure 8B, overlay). In sum, the pSpike area induced by co-activation of SuM and PP inputs was significantly larger than that of summated trace (Figure 8C, left). Notably, there was no significant change in the relative slope of fEPSP (Figure 8C, right). Further analysis of successive GC responses to either PP activation alone or co-activation of PP and SuM during the 5-Hz trains (Figure 8D, top traces) showed significant increases in the pSpike area (Figure 8D, bottom left plot), but not in the fEPSP slope (Figure 8D, bottom right plot). The lack of changes in the fEPSP slope during co-activation of PP and SuM supports the anatomical finding that SuM axons preferentially innervate the proximal part of GC dendrites.
We hypothesize that the excitatory effect of SuM activation on GCs could enhance LTP induction. To test this hypothesis, we stimulated the cortical input to GCs using a weak protocol (e.g., 20-Hz train stimulation) without and with SuM activation (Figure 8E). After train stimulation, we measured the changes in the synaptic responses. For the SuM + PP protocol, the electrical stimulation of the PP and photostimulation of the SuM input were timed to occur simultaneously ($\Delta t = 0$ ms; Figure 8E, left). The pSpikes were monitored after induction of LTP (Figure 8E). Notably, 20-Hz PP stimulation alone could not induce LTP (black circles); however, pairing it with photostimulation of the SuM input (20 Hz, 4 trains, 470 nm, 10 ms) resulted in an increase in pSpike and fEPSP slope (Figures 8F and G). Collectively, the SuM input enhanced GC responses to cortical inputs, thereby facilitating induction of LTP at the PP-GC synapses.
Glutamate and GABA are packed in distinct vesicles at the SuM terminals (Boulland et al., 2009; Root et al., 2018). Therefore, the loading, release, and recycling of these two neurotransmitters at the SuM terminals are likely to be regulated differentially. In this study, we demonstrated that glutamate/GABA co-releasing SuM neurons establish synapses with GCs and various subtypes of GABAergic INs in the DG. Notably, the synaptic excitation and inhibition at the SuM-DG synapses is target-specific. SuM-GC and SuM-S-IN synapses are predominantly GABAergic while SuM-D-IN synapses are mainly glutamatergic in nature.

The target cell-dependent excitation and inhibition at the SuM-DG synapses may be important for precise processing of neural information (Liu, 2004; Turrigiano and Nelson, 2004). We demonstrated a dominant inhibitory transmission at the SuM-S-IN synapses (Figure 3C), which might be responsible for weak disynaptic somatic inhibition in GCs (Hashimotodani et al., 2018). Feedforward inhibition is believed to enhance spike timing precision by curtailing EPSPs (Pouille and Scanziani, 2001). The reduced di-synaptic feedforward inhibition appears to be compensated by co-transmission of GABA along with glutamate at SuM-GC synapses. The imbalance of synaptic excitation and inhibition has been associated with neurological disorders, including epilepsy, autism spectrum disorders, schizophrenia, addiction, depression and social dysfunction (Meye et al., 2016; Shabel et al., 2014; Yizhar et al., 2011). Consistent with this notion, the SuM fibers in the supragranular layer extend aberrant axonal sprouting to the IML and are mostly VGlut2⁺ in an epileptic rat model (Soussi et al., 2015).

**A proposed modulatory role of SuM in the DG network**
Here, we proposed a network mechanism by which the SuM input modulates the input-output logic of the DG network (Figure 9). As shown by our experimental data, SuM neurons co-release glutamate and GABA. According to our study, S-INs receive greater synaptic inhibition than excitation (E < I), whereas D-INs receive stronger synaptic excitation than inhibition (E > I). Moreover, only D-INs generate spikes in response to SuM activation (Figure 9A), whereas S-INs respond with biphasic subthreshold potential changes (fast EPSP and slow IPSP). Our previous studies demonstrated that single AP generation in D-INs hardly triggers synaptic release onto GCs (Liu et al., 2014) and is therefore ineffective in modulating the GC output (Lee et al., 2016). Thus, SuM activation alone primarily causes small excitatory (red) and large inhibitory (blue) conductance changes around the somata of GCs (Figure 9A). As shown by our previous study (Chiang et al., 2012), GABA is depolarizing as the $E_{\text{GABA}}$ (approximately -72 mV) > resting membrane potential in GCs and could promote spike generation in GCs in response to the cortical input. The summation of the glutamate- and GABA-mediated conductances therefore results in subthreshold postsynaptic depolarization in GCs (Figure 9A). In great contrast to the SuM input, the PP input alone is sufficient to evoke spikes in S-INs (Lee et al., 2016; Liu et al., 2014). Accordingly, we propose that co-activation of SuM and PP inputs can trigger APs in both D-INs and S-INs (Figure 9B). Of note, D-INs and S-INs form reciprocal inhibition (Liu et al., 2014; Savanthrapadian et al., 2014; Scharfman et al., 1990; Sik et al., 1997). Thus, activation of the PP, SuM, and S-INs results in monosynaptic glutamatergic, mono-synaptic glutamatergic-GABAergic, and disynaptic somatic GABAergic conductance changes in GCs, respectively (Figure 9B). In line with our experimental data, the synaptic summation of these inputs results in AP generation in GCs (Figure 9B). During 20-Hz co-activation of the PP and SuM inputs, both D-INs and S-INs generate repetitive spikes (Figure 9C). Notably, D-INs dramatically increase their synaptic
output while they fire at burst frequency above 20 Hz (Liu et al., 2014). Accordingly, activation of the PP, SuM, S-INs, and D-INs result in mono-synaptic glutamatergic, mono-synaptic glutamatergic-GABAergic, disynaptic somatic, and disynaptic dendritic GABAergic conductance changes in GCs, respectively (Figure 9C). Overall, the synaptic summation of these inputs at 20 Hz results in multiple APs in GCs (Figure 9C), which is supported by our experimental data (Figure 8D). The enhanced spike generation in GCs during LTP induction is believed to be essential during the induction of Hebbian LTP.

After LTP induction, the pSpike was greatly enhanced (Figure 8F), whereas the fEPSP was modestly enhanced (Figure 8G). Although several potential mechanisms could account for these changes, a parsimonious explanation is the formation of Hebbian LTP (Figure 9D). Specifically, activity-dependent Hebbian LTP is accompanied by synaptic potentiation or a long-lasting increase in GC excitability as demonstrated by enhanced EPSP-spike (E-S) coupling (Figure 8F). Alternatively, the enhancement of E-S coupling after LTP induction could be mediated through network mechanisms. Given that the fEPSP at the PP-GC synapse was modestly increased (Figure 8H), we proposed that the D-IN-GC synapse may undergo weak long-term depression (iLTD), resulting a slight increase in the fEPSP (Figure 8G) after LTP induction. In contrast, the S-IN-GC synapse undergoes strong iLTD, resulting in a large decrease in somatic inhibition and therefore a large increase in the pSpike (Figure 8F). The future work is to investigate the changes in the synaptic efficacy at individual synapse in the DG circuits after LTP induction.
Cortical and subcortical afferents differentially recruit distinct types of DG INs

Extrinsic inputs differentially activate subtypes of GABAergic INs in the DG and play important roles in gating information transmission to the hippocampus (Armstrong et al., 2011; Chiang et al., 2012; Ewell and Jones, 2010; Hefft and Jonas, 2005; Hsu et al., 2016; Lee et al., 2016; Liu et al., 2014). We recently demonstrated that the commissural fibers of hilar MCs provide a strong excitatory drive to the S-INs, and D-INs, including ML cells and TML cells, while the medial PP (MPP) provides strong excitatory input to the S-INs (Hsu et al., 2016). In contrast, HIPP and HICAP cells receive weak excitatory inputs from the PP and are weakly recruited by the commissural fibers of hilar MCs (Hsu et al., 2016). This study revealed that activation of the SuM input alone can reliably recruit HIPP and HICAP cells. We have shown that both HIPP and HICAP cells dynamically regulate dendritic excitability of GCs (Liu et al., 2014). They weakly inhibit GCs when they fire sparsely, whereas they inhibit GCs robustly in the burst spiking mode (Liu et al., 2014). Overall, cortical and subcortical inputs may engage in hippocampal-dependent functions such as cognition and affective behaviors through differential recruitment of distinct types of DG INs.

SuM input differentially regulate inhibitory circuits

Although INs primarily innervate principal neurons, a growing body of evidence shows that DG INs connect and inhibit each other (Bartos et al., 2007; Liu et al., 2014; Wang and Buzsáki, 1996). Here, we show that the SuM input robustly recruit HIPP, TML, MOPP, and HICAP cells in the DG. These types of D-INs especially HIPP and HICAP are known to form synaptic connections with fast-spiking basket cells (BCs) (12.8% connectivity at HIPP-BC synapses and 16.3% connectivity at HICAP-BC synapses) and effectively inhibit spike generation and reduce spike jitters in BCs (Acsady et al., 2000; Savanthrapadian et al., 2014). Therefore, their direct or indirect activation could cause somatic disinhibition in
GCs and result in increased GC excitability. The DG ensembles are highly sensitive to the change of contextual cues (Danielson et al., 2016; Pignatelli et al., 2019). Somatostatin-expressing cells, including HIPP and TML cells, control the size of memory ensembles (Stefanelli et al., 2016). Therefore, activation of HIPP cells by the SuM input could regulate the size and specificity of memory engram.

GABAergic INs are believed to generate and maintain hippocampal theta activity (Freund, 2003; Freund and Buzsáki, 1996; Fricker and Miles, 2001; Ito et al., 2018; McBain and Fisahn, 2001). Given that the SuM plays an essential role in the generation and regulation of hippocampal theta activity, it would be interesting to determine the process by which D-INs are selectively recruited by SuM neurons in vivo. It will be more physiologically relevant to determine the process by which target cell-specific co-transmission of glutamate and GABA at the SuM-DG synapses contributes to brain computation in different behavioral states. The high excitation/low inhibition ($E > I$) at the SuM-D-IN synapses can promote dendritic inhibition, whereas the low excitation/high inhibition ($E < I$) at the SuM-GC synapses may help maintain minimal excitatory drive to GCs on one hand, and ensure high spiking precision on the other hand. The differential co-transmission of these two contrasting neurotransmitters at these two synapses may be crucial to the sparsity of GC activation, which plays a central role in pattern separation.

Correct representation of sensory information relies on the precise temporal firing of neurons (Kara et al., 2000; Reich et al., 1997; Reinagel and Reid, 2002). Here, we demonstrated that SuM-mediated glutamate-GABA co-transmission promotes spike-timing fidelity and reduces AP latency in GCs. This could be essential for ensuring the temporal precision of cognition and fidelity in separating barrage of sensory information into distinct outputs, as described in pattern separation. Moreover, the interaction among coincident inputs gives rise to associative plasticity and long-term regulation of information flow.
Consistent with this view, pairing the SuM input with the PP enhances the responses of GCs to cortical inputs, and also promotes long-lasting increase in the excitability of GCs. During LTP induction (Figure 8C), spikes are reliably generated in GCs. After the LTP induction, the PP-GC synapse is strengthened and there is a long-lasting increase in the excitability of GCs. In addition to synaptic summation, the observed net enhancement of GCs activity could be explained by IN network functions as illustrated in our proposed models (Figure 9). Given that fast-spiking BCs in the DG provide powerful inhibition onto GCs, suppression of their activities increases the response of GCs to the cortical input (Lee et al., 2016). Notably, dendritic inhibition driven by HIPP cells can reduce spike generation in BCs (Savanthrapadian et al, 2014). Our study showed that activation of the SuM input reliably excites HIPP and TML cells, which could suppress BCs activities, leading to somatic disinhibition of GCs and enhanced spike generation.
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Figure 1. Anatomical and physiological characterization of DG-projecting SuM neurons

(A) Left, schematic showing the location of retrogradely labeled cells in the SuM after bilateral red retrobead injections into the DG. Middle, representative images of injection sites along the anteroposterior (AP) axis of the DG. Right top, high magnification image of injection sites in the DG. Right bottom, retrogradely labeled DG-projecting SuM neurons in the SuM area.

(B) Schematic of unilateral red retrobead injection into the DG.

(C) Retrogradely labeled DG-projecting SuM neurons were mainly located in the right SuML ipsilateral to the injection site.

(D) Quantification of retrogradely labeled DG-projecting SuM neurons in the right and left SuML. Right SuML, 63 ± 4.2 cells; left SuML, 17 ± 3.5 cells; 12 slices from 3 mice; p < 0.0001, U = 2.0; Mann-Whitney test.

(E) IR-DIC image showing whole-cell recording from bead-positive neuron in the SuM.

(F) Representative firing pattern of a DG-projecting SuM neuron in response to 1-s current injection steps.

(G) Morphological reconstruction of a DG-projecting SuM neuron; soma and dendrites are depicted in black and axon in red. Black dotted lines depict boundary of the SuM area.

(H) Top, schematic of injection of AAV5-CaMKIIα-hChR2-eYFP into the SuM. Bottom, representative confocal image stacks of coronal section depicting ChR2-eYFP expression in the DG and CA2.

(I) Left, a biocytin-filled recording from a ChR2-expressing SuM neuron. Right, traces of light-evoked spikes recorded from the same cell in the presence of Kyn (2 mM), in current
clamp at -70 mV (top) and ChR2-mediated photocurrent recorded at approximately -70 mV in voltage clamp (bottom). Blue bars indicate the light pulses (5 ms, 470 nm, 5-Hz light pulse).

(J) Confocal image stacks of coronal section through the DG showing the projection pattern of SuM terminals in the DG. DAPI (left), ChR2-expressing SuM terminals (middle) and merged image (right).

(K) Confocal image stacks of SuM axon terminals expressing ChR2-eYFP, VGlut2, VGAT immunofluorescence and the merge image showing their co-localization on the labeled SuM terminals. Right, putative boutons in the box.

Figure 2. SuM input preferentially excites dendrite-targeting INs in the DG

(A) Top, experimental configuration of local field potential (LFP) recordings and photostimulation. A transverse section across the DG showing ChR2-eYFP-expressing SuM fibers (green) in the GCL and light-evoked LFPs recorded along the somatodendritic axis of GCs in the DG. Bottom, schematic of local network of the DG depicting GC (gray), S-IN (orange) and D-IN (violet).

(B-D) Top, representative morphological reconstruction of a GC, a S-IN and a D-IN (soma and dendrites, black; axon, red) in the DG. Middle, sample traces of cell-attached responses (six overlaid sweeps) to 5-Hz photostimulation of the SuM input and firing pattern of a representative GC, S-IN and D-IN. Bottom, plot of spike probabilities of all recorded cells.

(E) Summary of identified D-INs subtypes recruited by the SuM input are depicted. The filled circles (soma locations), the thick lines (dendrites) and the hatched boxes (axon distribution).
(F) Top, morphological reconstructions of representative TML, HIPP, MOPP and HICAP in the DG. Bottom, plot of spike probabilities of recorded cells in response to 5-Hz photostimulation of the SuM input. Data error bars represent mean ± SEM.

**Figure 3. Differential glutamate/GABA co-transmission is target cell-specific**

(A) Top, schematic of virus injection into SuM of VGluT2-Cre or WT mouse. Bottom, schematic of local DG network including the SuM input (green), GC, S-IN and D-IN.

(B), (C), and (D) Top, confocal image stacks of transverse sections of the DG depicting selective expression of ChR2-eYFP in the GCL and a biocytin-filled GC, S-IN and D-IN (red). Middle, firing pattern of the GC, S-IN and D-IN. Bottom, sample traces showing the responses of a GC, S-IN and D-IN to the 5-Hz photostimulation of the SuM input. Black traces; average inward currents recorded in ACSF, in the presence of GABA\_A receptor blocker, SR95531 (1 μM, SR) and GABA\_B receptor blocker, CGP55845 (1 μM, CGP), and in the presence of SR, CGP and 2 mM Kyn. The Kyn-sensitive component (glutamatergic, red), and SR & CGP-sensitive component (GABAergic, blue) obtained by digital subtraction from the above traces.

(E) Plot of synaptic latencies of EPSC\(_1\) and IPSC\(_1\) induced by the first light pulse in GCs, S-INs and D-INs. (GCs, EPSC\(_1\), 2.60 ± 0.10 ms; IPSC\(_1\), 2.56 ± 0.10 ms; n = 30; p = 0.875, U = 439.0; S-INs, EPSC\(_1\), 2.78 ± 0.20 ms; IPSC\(_1\), 3.07 ± 0.23 ms; n = 6; p = 0.571, U = 14.0; D-IN, EPSC\(_1\), 2.67 ± 0.09; IPSC\(_1\), 2.73 ± 0.10; n = 22; p = 0.663, U = 223.0; Mann-Whitney test. Circles connected by lines represent data collected from the same cell. Filled circles are data obtained from VGluT2-Cre line while open circles represent data from WT mice.

(F) Plot of excitatory and inhibitory conductances, EPSG\(_1\) and IPSG\(_1\) in GCs, S-INs and D-INs. (GCs, EPSG\(_1\), 0.30 ± 0.03; IPSG\(_1\), 0.91 ± 0.08; n = 30; p < 0.0001; U = 67.0; S-INs,
EPSG\(_1\), 0.58 ± 0.08 nS; IPSG\(_1\), 2.14 ± 0.67 nS; n = 6; p < 0.05; U = 4.0; D-INs, EPSG\(_1\), 0.99 ± 0.08 nS; IPSG\(_1\), 0.48 ± 0.08 nS; n = 22; p < 0.0001; U = 63; Mann-Whitney test. 

(G) Scatter plot of EPSG\(_1\) versus IPSG\(_1\) from GCs (gray circles), S-INs (orange circles) and D-INs (violet circles). The dashed line represents equality diagonal. The gray, orange and violet lines are the linear regression lines for GCs, S-IN and D-INs respectively (the slope = 0.34; \(R^2 = 0.20\) for GCs, slope = 0.17, \(R^2 = 0.68\) for S-INs, and slope = 1.24, \(R^2 = 0.68\) for D-INs). Data error bars represent mean ± SEM. 

**Figure 4. SuM input forms monosynaptic connections with GCs and D-INs**

(A) Schematic of virus injection into the SuM of VGluT2-Cre mice.

(B) Representative traces of light-evoked responses recorded from a GC in ACSF, TTX (1 μM), TTX, 4-AP (1 mM). Note that TTX completely block the response and recovered by 4-AP. Addition of SR (1 μM) and CGP (1 μM) largely block the response, Kyn (2 mM) completely abolished the remaining responses.

(C) Synaptic latencies before and after bath application of TTX, 4-AP at the SuM-GC synapse; ACSF, 2.24 ± 0.11 ms; TTX, 4-AP, 4.01 ± 0.28 ms; n = 9; p = 0.0039, Wilcoxon sign-rank test.

(D) Plot of EPSG\(_1\) and IPSG\(_1\) of GCs. EPSG\(_1\), 0.53 ± 0.10 nS; IPSG\(_1\), 1.95 ± 0.51 nS; n = 9; p = 0.0012; U = 6.0; Mann-Whitney test.

(E) Representative traces of light-evoked responses recorded from a D-IN in ACSF, TTX (1 μM), TTX, 4-AP (1 mM). TTX completely block the response and recovered by 4-AP. SR (1 μM) and CGP (1 μM) slightly block the response, and finally, Kyn (2 mM) completely abolished the remaining responses.
(F) Plot of synaptic latencies before and after bath application of TTX, 4-AP at the SuM-D-IN synapses; ACSF, $2.67 \pm 0.21$ ms; TTX & 4-AP, $3.66 \pm 0.17$ ms; $n = 6$, $p = 0.0313$, Wilcoxon sign-rank test.

(G) Plot of EPSG$_1$ and IPSG$_1$ of D-INs. EPSG$_1$, $2.16 \pm 0.51$ nS; IPSG$_1$, $0.95 \pm 0.06$ nS; $n = 6$; $p = 0.0411$; $U = 5.0$; Mann-Whitney test.

(H) Scatter plot of EPSG versus IPSG from GCs (gray circles) and D-INs (violet circles) during 5-Hz photostimulation of SuM input. The dashed line represents equality diagonal. The gray and violet lines are the linear regression lines for GCs and D-INs, respectively (the slope = 0.14; $R^2 = 0.40$ for GCs and slope = 1.40, $R^2 = 0.78$ for D-INs). Data error bars represent mean ± SEM.

(I) Schematic of virus injection into the SuM of VGAT-Cre (open circle) and Gad2-Cre (closed circle) mice.

(J) Sample traces showing the responses of a GC to the 5-Hz photostimulation of the SuM input. Black trace; average inward currents recorded in ACSF, in the presence of GABA$_A$ receptor blocker, SR95531 (1 μM, SR) and GABA$_B$ receptor blocker, CGP55845 (1 μM, CGP), and in the presence of SR, CGP and 2 mM Kyn. The Kyn-sensitive component (glutamatergic, red), and SR & CGP-sensitive component (GABAergic, blue) obtained by digital subtraction from the above traces.

(K) Plot of synaptic latencies of EPSC$_1$ and IPSC$_1$ of GCs. EPSC$_1$, $2.91 \pm 0.14$ nS; IPSC$_1$, $2.86 \pm 0.14$ nS; $n = 8$; $p = 0.5604$; $U = 26.0$; Mann-Whitney test.

(L) Plot of conductances EPSG$_1$ and IPSG$_1$ of GCs. EPSG$_1$, $0.20 \pm 0.03$ nS; IPSG$_1$, $0.48 \pm 0.06$ nS; $n = 8$; $p = 0.0006$; $U = 2.0$; Mann-Whitney test.

**Figure 5.** MCs receive weak synaptic input from the SuM
(A) Left, confocal image stacks of transverse sections through the DG depicting selective expression of ChR2-eYFP in VGluT2<sup>+</sup> SuM fibers (green) in the GCL and sequentially recorded biocytin-filled MC #1, MC #2 (arrow heads, thorny excrescences), and a GC. Right, representative traces obtained from MC #1, MC #2 and a GC in response to the photostimulation of the SuM input.

(B) Left, morphology of a biocytin-filled responsive MC #3. Right, black traces, individual traces of responses of the MC #3 to 5 Hz photostimulation of SuM input. The red trace is the average trace. The arrows denote disynaptic responses.

(C) and (D) Summary of the EPSG<sub>1</sub> and IPSG<sub>1</sub> respectively, recorded from different cell types in the DG. Individual cells were shown in circles. Data error bars represent mean ± SEM.

Figure 6. Synaptic responses from simultaneously recorded GCs and D-INs

(A) Schematic of virus injection into the SuM.

(B) Left, simultaneous whole-cell recording from a GC and a D-IN. Middle, the firing pattern of the recorded GC and D-IN. Right, the morphological reconstruction GC (gray color) and D-IN (violet color).

(C) Traces of light-evoked postsynaptic responses recorded in GC and D-IN in baseline, SR & CGP, SR, GCP & Kyn, glutamatergic component (red), and GABAergic component (blue).

(D) Plot of the total composite current amplitude in the GCs and D-INs simultaneously recorded. GC, 43.94 ± 9.24 pA; D-IN, 89.78 ± 21.13 pA; n = 6; p = 0.0931; U = 7.0; Mann-Whitney test. Circles connected by dashed lines represent data collected from cells recorded simultaneously from the same slice.
(E) Plot of conductances of EPSG and IPSG at the SuM-GC and SuM-D-IN synapses.

SuM-GC, EPSG$_1$, 0.22 ± 0.05 nS; IPSG$_1$, 0.52 ± 0.10 nS; n = 5; p < 0.05; U = 2.0; SuM-D-IN, EPSG$_1$, 1.24 ± 0.26 nS; IPSG$_1$, 0.40 ± 0.09 nS; n = 5; p < 0.01; U = 0.0; Mann-Whitney test.

**Figure 7. SuM input shortens spike latency and enhances spike-timing precision**

(A) Top, representative traces of responses of GCs to sinusoidal current steps before (left) and after (right) photostimulation of SuM input. Middle, baseline to peak current amplitude of 100 pA sinusoidal protocol (red traces). Bottom, the EPSP evoked by photostimulation of SuM input. Gray bars represent light off while blue bars indicate time of photostimulation at 5 Hz.

(B) Plot of spike number versus baseline to peak current in GCs.

(C) Top, representative traces of responses of D-INs to sinusoidal current steps before (left) and after (right) photostimulation of SuM input. Middle, baseline to peak current amplitude of 110 pA sinusoidal protocol (red traces). Bottom, the EPSP evoked by photostimulation of SuM input. Blue bars indicate time of photostimulation at 5 Hz.

(D) Plot of spike number versus baseline to peak current in D-INs.

(E) Representative traces of responses of GCs (twenty overlaid sweeps) to constant suprathreshold sinusoidal current injection without (upper traces) and with (lower traces) photostimulation of SuM input. Left, enlarged traces of APs induced by first stimulus without (upper traces) and with photostimulation of SuM input (lower traces). Red dotted lines and the red arrow lines show a shift in the mean spike latencies between onset of sinusoid current injection and the mean time point of peak in each AP. The pink bars represent spike jitters.
(F) Summary plot of spike phase. $n = 12; F(4, 44) = 20.43; p < 0.0001; \text{n.s., no significant difference}; \text{two-way ANOVA with Bonferroni post hoc test. Data error bars represent mean ± SEM.}$

(G) Summary plot of spike jitter. $n = 12; F(4, 44) = 22.17; p < 0.0001; \text{n.s., no significant difference}; \text{two-way ANOVA with Bonferroni post hoc test.}$

(H) Representative traces of responses of D-INs (twenty overlaid sweeps) to constant suprathreshold sinusoidal current injection without (upper traces) and with (lower traces) photostimulation of the SuM input. Left, enlarged traces of APs induced by first stimulus without (upper traces) and with photostimulation (lower traces). Red dotted lines and the red arrow lines show a shift in the mean spike latencies between onset of sinusoid current injection and the mean time point of peak in each AP. The pink bars represent spike jitters.

(I) Summary plot of spike phase. $n = 10; F(4, 36) = 115.4; p < 0.0001; \text{two-way ANOVA with Bonferroni post hoc test. Data error bars represent mean ± SEM.}$

(J) Summary plot of spike jitter. $n = 10; F(4, 36) = 5.0; p = 0.0027; \text{n.s., no significant difference}; \text{two-way ANOVA with Bonferroni post hoc test.}$

**Figure 8. SuM input promotes GC responses to cortical input, thereby enhancing LTP at the PP-GC synapses**

(A) Experimental schematic showing a stimulation electrode (stim.) placed in the subiculum to electrically activate the PP fibers, a field-recording electrode in the GCL to monitor LFP and pSpike, and a blue light for photostimulation of the SuM axon terminals in the GCL.

(B) Representative traces of SuM-mediated fEPSP (black trace) after photostimulation, PP-mediated pSpike (filled area in gray) upon electrical stimulation, and a pSpike (filled
area in light blue) after the co-activation (∆t = 0 ms) of the SuM and PP. The arithmetic sum of fEPSP and pSpike was shown in red. The traces of pSpikes were superimposed and aligned with fEPSP.

(C) Left bar graph, summary plots of the pSpike areas evoked by SuM+PP co-activation (light blue) and arithmetic sum of SuM-evoked fEPSP and PP-evoked pSpike (light red). Areas were normalized to pSpike area evoked by the PP alone. SuM+PP co-activation, 1.43 ± 0.16; SuM+PP arithmetic sum, 1.17 ± 0.05; n = 6; p = 0.0313. Right bar graph, summary plots of relative fEPSP slope, SuM+PP co-activation, 1.01 ± 0.02; SuM+PP arithmetic sum, 0.97 ± 0.01; n = 6; n.s., no significant difference; Wilcoxon signed-rank test.

(D) Top, representative traces of pSpike responses to PP stimulation alone (black traces) and SuM+PP (blue traces) during a 5-Hz trains. Bottom, left, summary of the effect of SuM activation on PP-evoked pSpikes versus stimulus number. PP, n = 6; PP + SuM, n = 6; p < 0.05; two-way ANOVA with Bonferroni post hoc test. Right, fEPSP slope before and after photostimulation of the SuM input. PP, n = 6; SuM+PP, n = 6; n.s., no significant difference, two-way ANOVA with Bonferroni post hoc test.

(E) Left, representative traces of baseline pSpikes in response to stimulation of PP alone. Middle, LTP induction protocol consisting of four trains of 20-Hz electrical stimulation of the PP alone at 15 s inter-train interval (top) or co-activation of the PP and 20-Hz, 4 trains, 10 ms photostimulation of the SuM input (bottom). Right, sample traces of pSpikes after LTP induction.

(F) Time course of the normalized pSpike area recorded from the GCL in response to 20-Hz, 4 trains stimulation of PP inputs alone (black circles) or co-activation of the PP input stimulation and 20-Hz photostimulation of the SuM input (blue circles). PP alone, 104.8 ± 8.59%; n = 6; SuM+PP, 167.6 ± 5.30%; n = 6; p = 0.0009; paired t-test.
(G) Time course of the normalized fEPSP slope of pSpikes recorded from the GCL in response to 20-Hz, 4 trains stimulation of PP inputs alone (black circles) or co-activation of PP input stimulation with 20-Hz photostimulation of the SuM input (blue circles). PP alone, 110.6 ± 2.20%; n = 6; SuM+PP, 128.5 ± 5.19%; n = 6; p = 0.0598; paired t-test. Data error bars represent mean ± SEM.

Figure 9. A proposed modulatory role of SuM input in the DG network

(A) Schematic of the DG network model showing the synapses between the SuM input (green) and the GC (gray), the D-IN (violet square), and S-IN (orange oval). The SuM input forms monosynaptic excitatory and inhibitory connections with the GC, D-IN and S-IN. At SuM-GC and SuM-S-IN, E<I while at SuM-D-IN, E>I. Activation of SuM input (green AP) results in spike generation in D-IN (violet AP), but only subthreshold depolarization in the GCs and S-INs. The synaptic summation in this model leads to a small subthreshold depolarization in the GCs.

(B) Co-activation of the SuM input (green) and PP input (red). The spike generation in the D-IN (violet AP) is reinforced by PP stimulation. S-IN is recruited into the network by the PP input (orange AP). The summation of the synapses results in enhanced EPSP (E)-spike (S) coupling (gray E-S coupling) in the GC.

(C) Co-activation of SuM and PP inputs during LTP induction. 20 Hz simultaneous activation of SuM (green spikes) and PP (red spikes). During this LTP induction protocol, spike generation in D-IN is strongly reinforced through the entire phase of the stimulation trains while S-IN generates spikes only at the early phase (orange spikes); this could result in a late somatic disinhibition of GC. The synaptic summation during this induction protocol leads to net increase in spike generation in GC (gray spikes).
(D) Synaptic output by PP activation alone after LTP induction. Both PP-GC synapse and E-S coupling are enhanced.
