Input integration around the dendritic branches in hippocampal dentate granule cells

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Abstract Recent studies have shown that the dendrites of several neurons are not simple translators but are crucial facilitators of excitatory postsynaptic potential (EPSP) propagation and summation of synaptic inputs to compensate for inherent voltage attenuation. Granule cells (GCs) are located at the gateway for valuable information arriving at the hippocampus from the entorhinal cortex. However, the underlying mechanisms of information integration along the dendrites of GCs in the hippocampus are still unclear. In this study, we investigated the input integration around dendritic branches of GCs in the rat hippocampus. We applied differential spatiotemporal stimulations to the dendrites using a high-speed glutamate-uncaging laser. Our results showed that when two sites close to and equidistant from a branching point were simultaneously stimulated, a nonlinear summation of EPSPs was observed at the soma. In addition, nonlinear summation (facilitation) depended on the stimulus location and was significantly blocked by the application of a voltage-dependent Ca^{2+} channel antagonist. These findings suggest that the nonlinear summation of EPSPs around the dendritic branches of hippocampal GCs is a result of

voltage-dependent Ca^{2+} channel activation and may play a crucial role in the integration of input information.

Keywords Hippocampus · Dendrite · Excitatory postsynaptic potentials summation · Uncaging · Supralinear amplification

Introduction

Dentate granule cells (GCs) are located in the dentate gyrus, the entrance to the hippocampal trisynaptic network. GCs mainly receive synaptic inputs through the perforant path (PP) that originates in the entorhinal cortex (Amaral et al. 2007). The lateral PP projects to distal dendrites and the medial PP projects to middle dendrites within the outer two-thirds of the GC molecular layer (Wang and Lambert 2003; Nishimura-Akiyoshi et al. 2007; Hargreaves et al. 2005), while associational–commissural inputs and positive feedback inputs from mossy cells terminate on proximal dendrites within the inner third molecular layer (Jackson and Scharfman 1996). It is conceivable, therefore, that information processing differs at each dendritic location.

GCs differ from hippocampal pyramidal cells morphologically as they have profuse branches not far from the soma within the inner third of the molecular layer (Amaral et al. 2007). Moreover, due to very strong dendritic voltage attenuation, the impact of individual synapses on GC output is low (Krueppel et al. 2011). It was previously thought that dendrites simply collect synaptic inputs and convey them to the soma, but recent studies have reported that dendrites can serve as fundamental units integrating some information by themselves. For example, sequential spine activation from the tip branch to the soma (afferent

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direction) or from the soma to the tip (efferent direction) produces strong direction-sensitive responses, where the afferent direction consistently produces larger somatic responses than the efferent direction in dentate GCs and where the response is dependent on *N*-methyl-D-aspartate (NMDA) receptors (Branco and Häusser 2010). The facilitation of dendritic inputs with direction sensitivity may therefore be crucial in dendritic information integration. In our previous study, we demonstrated the nonlinear integration of spatiotemporal inputs to dendrites in CA1 pyramidal neurons using laser uncaging stimulation (Yoneyama et al. 2011). However, not just the dendrites but also their single dendritic branches may act as fundamental functional units in the nervous system (Branco et al. 2010), given that GC dendrites show linear integration of synaptic inputs, because the strong voltage attenuation along the dendrites regulates and prevents induction of nonlinear summation. However, since GCs contain numerous branches and specific input locations with different information modalities (Hargreaves et al. 2005), the mechanisms underlying the integration of synaptic inputs involving dendritic branching, the interaction between these inputs along the dendrite, and the integration of information from inputs at the dendrite tufts are still unclear.

In this study, we focused our investigation on input integration that occurs around the dendritic branches of GCs. Using a high-speed glutamate-uncaging laser, we applied differential spatiotemporal stimulation to the dendrites to estimate the linearity of summation of excitatory postsynaptic potentials (EPSPs) by two dendritic inputs around the branching point.

Materials and methods

All experiments were performed in accordance with the guidelines of Tamagawa University Animal Care and Use Committee. The protocol was approved by the Tamagawa University Animal Care and Use Committee (Permit Number: H14-8).

Animals and brain slices

Hippocampal acute slices were prepared from Wistar rats (P18–22). Rats were anesthetized with Forane (Abbott Japan). The whole brain was removed while under anesthesia and placed in cold artificial cerebrospinal fluid (ACSF) containing (in mM) 124 NaCl, 3 KCl, 10 glucose, 1.25 NaH₂PO₄, 22 NaHCO₃, 2 MgSO₄, and 2.5 CaCl₂ (pH 7.3 when bubbled with 95 % O₂ and 5 % CO₂). The brain was sliced (300- μ m thickness) at an angle of 30°–40° along the long axis of the hippocampus. Acute slices were recovered in ACSF at 30 $\pm$ 0.5 °C for at least 1 h before

recording. After recovery, the slices were kept at room temperature. During recording, 25 μ M of picrotoxin (Sigma-Aldrich) was added to the ACSF to block GABA_A receptor-mediated current.

Stimulation with high-speed uncaging equipment

Stimulation was performed using a laser confocal microscope with a custom built high-speed UV laser uncaging system (Carl Zeiss) (Kojima 2006). The system can irradiate a sample with a radius less than 1 μ m. To visualize the dendrites, Alexa Fluor 488 (100 μ M; Molecular Probes) was added to the pipette solution and applied to the cell for 10 min. The dendrite was visualized using an argon laser (488 nm) with a confocal laser scan microscope. Before laser uncaging stimulation, MNI-caged glutamate (125 μ M final concentration; Tocris) was added to the ACSF. The UV laser irradiated the dendrite at the outer molecular layer in the upper blade of the dentate gyrus. The duration of laser irradiation was 1 ms, and laser power was adjusted so that the peak amplitude of unitary EPSP was 1.5–2.5 mV. The unitary EPSPs were adjusted to below the threshold. The stimulus interval (τ) was set as the time from the start of laser irradiation to the next laser irradiation. Due to limitations of the uncaging system used, $\tau = 0$ ms had an interval delay of 0.5–1 ms in transit time. The other intervals did not have any delay because of correction. In addition, in the hippocampal dentate gyrus, regardless of the location of the dendrite, the rise time of glutamate-uncaging-induced EPSPs (gluEPSPs) at a single spine is stable, and the decay time does not change with DL-2-amino-5-phosphonovaleric acid or Ni²⁺ administration (Krueppel et al. 2011).

Superposed EPSP around a branching point

To investigate the spatiotemporal properties of EPSP summation on the dendrite, stimulus locations were set to three sites along the dendrite or dendritic branches (Fig. 1a). Two stimulations S_1 and S_2 (Fig. 1b) were applied to sites 1 and 2 on branching tufts, located at distances d_1 and d_2 from the dendritic branching point, respectively ($d_1 = d_2$; 5, 10, 20, 30 μ m). Another stimulation, S_3 , was applied to site 3 on the apical dendrite, located at distance d_3 from the chosen dendritic branch to the soma (d_3 ; 5, 10 μ m). A pairing stimulation S_{ij} was applied (Fig. 1c), consisting of stimulations S_i and S_j with interval time τ (0, 5, 10 ms). A pairing stimulation consisting of S_1 (or S_2) on a tuft and S_3 on the apical dendrite (Fig. 1d, left) is hereafter referred to as “Line” stimulation S_{13} and S_{23} . S_{13} and S_{23} indicate the direction from the tip to the soma, which we refer to as the “IN” direction. S_{31} and S_{32} indicate the direction from the soma to the tip,

Fig. 1 Experimental method and measurement. **a** Left: example imaging of a dentate GC filled with Alexa Fluor 488. Rectangular box indicates dendrites selected for experiment. Right: selected dendrites with three glutamate uncaging spots (1, 2, and 3), which are expanded in the rectangular box on the left. Stimulus spots S_1 and S_2 are divided on two daughter dendrites. Scale bar 10 μm . **b** Locations of three stimulus sites (1, 2, and 3) around a dendritic branching point. d_1 , d_2 , d_3 : distance from branching point to stimulus site. d_4 : distance from soma to branching point. **c** Pairing stimulation. S_i , S_j : single stimulation to sites i , j ($i, j = 1, 2, 3$). S_{ij} : pairing stimulation consisting of S_i preceding S_j with interval time $\tau = 0, 5,$

10 ms). **d** Two types of pairing stimulation. Left: Line stimulations S_{13} and S_{23} consisting of a stimulus to site 1 or 2 and stimulation S_3 to site 3. S_{13} and S_{23} with S_1 or S_2 preceding S_3 are referred to as being in the “IN” direction. S_{31} and S_{32} with S_3 preceding S_1 or S_2 are referred to as being in the “OUT” direction. Left: Branch stimulation S_{12} and S_{21} consisting of stimuli to sites 1 and 2. **e** The measurement of the nonlinearity in the EPSP summation induced by pairing stimulation. Upper and middle traces: EPSP time course for a single stimulation S_i and S_j . Lower traces: measured EPSP for pairing stimulation (bold trace) and linear summation of EPSP time course for S_1 and S_2 stimulations (gray trace)

which we refer to as the “OUT” direction. Another pairing stimulation consisting of S_1 and S_2 on each tuft, or sister branch, (right part of Fig. 1d) is hereafter referred to as

“Branch” stimulation S_{12} . S_{12} and S_{21} represent pairing stimulations for the same pair of sites in opposite directions. A stimulus was defined as such when S_{12} induced a

greater superposed EPSP than S_{21} ($d_1, d_2 = 10 \mu\text{m}$, $\tau = 5 \text{ ms}$). The measurement of nonlinearity in the EPSP summation induced by pairing stimulation is shown in Fig. 1e. In the lower traces, nonlinearity was calculated as the difference between the measured (superposed) EPSP elicited for pairing stimulation S_{12} and the linear summation of each EPSP time course for S_1 and S_2 stimulations (upper and middle traces).

Recording

Patch clamp recording was performed at the soma of a dentate GC from rat hippocampus. The recording electrode was a micro glass pipette made with a puller. The resistance of the electrode was 7–10 M Ω . The pipette solution contained (in mM) 142 K-glucuronate, 10 HEPES, 10 NaCl, 2 MgATP, 0.2 Na₂GTP, 0.5 EGTA, and 10 MgCl₂ (pH 7.2 with KOH). To visualize the dendrite, Alexa Fluor 488 was added to the pipette solution (final concentration was 100 μM). Before laser uncaging stimulation, MNI-caged glutamate (125 μM final concentration; Tocris) was applied to the ACSF. During recording, 25 μM of picrotoxin (Sigma-Aldrich) was added to the ACSF to block GABA_A receptor-mediated current. Neuronal responses were recorded using the whole-cell patch clamp method (current-clamp mode). In our pharmacological experiment (described later), the blockers were applied to the ACSF bath. A patch clamp amplifier (EPC-7 plus; Heka) was used for the recording. The starting membrane potential of the GCs used for our experiments was less than -60 mV. The membrane potential was adjusted to -80 mV by current injection. The neural response was high-cut filtered at 5 kHz and digitized at 48 kHz using personal computer software (Clampex 9.2.0.11, Molecular Devices).

Experiment 1: spatial dependence of EPSP summation

To clarify the spatial dependence of the linearity or nonlinearity in an EPSP summation induced by coincident applications of two inputs, *Line* stimulations (S_{13}, S_{23}) and *Branch* stimulations (S_{12}, S_{21}) were applied with the same time interval $\tau = 0 \text{ ms}$ at the same distances ($d_1, d_2 = 5, 10, 20, 30 \mu\text{m}$; $d_3 = 5, 10 \mu\text{m}$). In addition, the dependence of the nonlinearity on the distance between a branching point and the soma (d_s) was measured for the above pairing stimulations in which significant nonlinearity in EPSP summation was observed.

Experiment 2: spatiotemporal dependence of EPSP summation

To clarify the spatiotemporal dependence of the linearity or nonlinearity in an EPSP summation, *Line* stimulations (*IN*:

S_{13}, S_{23} and *OUT*: S_{31}, S_{32}) and *Branch* stimulations (S_{12}, S_{21}) were applied at different time intervals ($\tau = 0, 5, 10 \text{ ms}$) and at the same distances ($d_1, d_2 = 5, 10 \mu\text{m}$ and $d_3 = 5, 10 \mu\text{m}$).

Pharmacological application

To clarify the molecular mechanism underlying the non-linear summation of EPSP on dendrites, we applied two antagonists to the ACSF: DL-2-amino-5-phosphonopentanoic acid (DL-AP5, 100 μM , Sigma-Aldrich) for the NMDA receptor, and NiCl₂ (50 μM , Kanto Chemicals) for the voltage-dependent Ca²⁺ channel.

Analysis

All data processing was performed after applying a 1-kHz low pass filter (Clampfit ver. 9.2.0.11; Molecular Devices). For a single neuron, five responses to the paired stimuli were averaged and used as representative data. The 50 ms of resting membrane potential before stimulation was averaged and defined as 0 mV. To evaluate the nonlinearity of dendritic EPSP summation, we calculated the ratio between the peak of the measured EPSP and the peak of the EPSP linear sum (Fig. 1e). T-tests and ANOVAs were used to determine statistical significance as appropriate, with significance set at $P < 0.05$.

Results

Spatial dependence of EPSP summation

To determine the existence of nonlinearity in an EPSP summation induced by coincident application of two inputs on dendrites, *Line* stimulations (S_{13}, S_{23}) and *Branch* stimulations (S_{12}, S_{21}) were applied with a time interval $\tau = 0 \text{ ms}$ at the same distances from a branching point ($d_1, d_2 = 5, 10, 20, 30 \mu\text{m}$ and $d_3 = 5, 10 \mu\text{m}$). First, in Fig. 2, we show the comparison between the measured EPSP and the EPSP linear summation around a dendritic branch when the pairing stimulations were applied with $\tau = 0 \text{ ms}$ at the same distance of 10 μm from the branching point ($d_1 = d_2 = d_3$). In Fig. 2a, linearity is shown in the input–output relation for each *Line* stimulation (S_{13}, S_{23}). Figure 2b shows summarized data of their input–output relation for each distance from the branching point. Results showed that there was no nonlinearity in the input–output relations as distance between stimulus point and branching point increased (Student's t test, $P < 0.05$; $d_1, d_2, d_3 = 5 \mu\text{m}$, S_{13} : 1.11 ± 0.08 ($n = 7$), S_{23} : 1.08 ± 0.10 ($n = 7$); $d_1, d_2, d_3 = 10 \mu\text{m}$, S_{13} : 1.04 ± 0.04 ($n = 5$), S_{23} : 1.03 ± 0.04 ($n = 5$); $d_1, d_2, d_3 = 20 \mu\text{m}$, S_{13} : 0.97 ± 0.02 ($n = 8$), S_{23} :

Fig. 2 Spatial dependence of EPSP summation in *Line* stimulation. EPSP summations induced by coincident applications of pairing inputs on dendrites of *Line* stimulations (S_{13} , S_{23}). **a** Input–output relation between the measured EPSP and the EPSP linear summation are plotted for when the pairing stimulus S_{12} and S_{23} was applied with the time interval $\tau = 0$ ms and at the same distance of 10 μm from branching point to stimulation site ($d_1 = d_2 = d_3$). **b** The ratio of the measured EPSP and the EPSP linear summation was calculated as the input–output relation by *Line* stimulations (S_{13} , S_{23}) with the time interval $\tau = 0$ ms and at the same distances from the branching point (d_1 , d_2 , $d_3 = 5$, 10, 20 μm). There was no significant nonlinear summation of EPSP. Maximum distance from soma to branching point was approximately 180 μm . Values in **b** are given as mean $\pm$ SE

0.97 ± 0.05 ($n = 7$)). Figure 3 shows the input–output relation when the *Branch* stimulations (S_{12} or S_{21}) were applied coincidentally ($\tau = 0$ ms) at the same distances (d_1 , $d_2 = 5$, 10, 20, 30 μm). In Fig. 3a, most measured EPSPs were greater than the expected EPSP linear sums for each *Branch* stimulus (S_{12}) at 10 μm from branching point to stimulus point, regardless of the magnitude of the input. Figure 3b shows summarized data of the input–output relation for each distance. Significant nonlinearity was found for the 5 and 10 μm distances (Student's *t*-test, $P < 0.05$; d_1 , $d_2 = 5$, 1.24 ± 0.06 ($n = 7$) $P < 0.01$; d_1 , $d_2 = 10$ μm , 1.26 ± 0.03 ($n = 69$)). Significant nonlinearity was not found for the longer 20 and 30 μm distances (d_1 , $d_2 = 20$ μm , 1.09 ± 0.03 ($n = 26$); d_1 , $d_2 = 30$ μm , 1.02 ± 0.04 ($n = 7$)). When comparing nonlinearity at different distances, significant differences were found between

Fig. 3 Spatial dependence of EPSP summation in *Branch* stimulation. EPSP summations induced by coincident applications of pairing inputs on dendrites of *Branch* stimulations (S_{12} or S_{21}). **a** Input–output relation between the measured EPSP and the EPSP linear summation are plotted for when the pairing stimulus S_{12} ($= S_{21}$) was applied with the time interval $\tau = 0$ ms and at the same 10 μm distance from branching point to stimulation site ($d_1 = d_2$). **b** The ratio of the measured EPSP and the EPSP linear summation was calculated as the input–output relation by *Branch* stimulations S_{12} ($= S_{21}$) with the time interval $\tau = 0$ ms and at the same distances from the branching point (d_1 , $d_2 = 5$, 10, 20, 30 μm). Significant nonlinearity was found for 5 and 10 μm distances (ANOVA, $P < 0.05$), and the supralinearity gradually decreased with increasing distance. Maximum distance from soma to branching point was approximately 180 μm . Values in **b** are given as mean $\pm$ SE

the 5 and 30 μm distances, 10 and 20 μm distances, and 10 and 30 μm distances (ANOVA, $P < 0.05$). Therefore, supralinearity gradually decreased with increasing distance.

In addition, in Fig. 4, the dependence of the nonlinearity on the distance d_s of a branching point from the soma was measured for *Branch* stimulation S_{12} ($d_1 = d_2 = 10$ μm). Here, significant nonlinearity in the EPSP summation was observed. The increasing ratios presenting nonlinearity stabilized at less than 100 μm from soma to branching point.

Spatiotemporal dependence of EPSP summation

To determine the spatiotemporal dependence of the linearity or nonlinearity in EPSP summation, *Line* stimulations (*IN*: S_{13} , S_{23} and *OUT*: S_{31} , S_{32}) and *Branch* stimulations (S_{12} , S_{21}) were applied at different timings ($\tau = 0$, 5, 10 ms) and at the same distances (d_1 , $d_2 = 5$, 10 μm).

Results for *Line* stimulations applied at different timings ($\tau = 0$ and 10 ms) and at the same distance (d_1 , d_2 , $d_3 = 10$ μm) are shown in Fig. 5. In the present study, the stimulus sites 1 and 2 for S_1 and S_2 in the *Line* stimulation were decided as such when the superposed EPSP elicited by S_{13} was larger than that by S_{23} . Although there was no

Fig. 4 EPSP summation dependence on distance from the soma to the branching point in *Branch* stimulation. The dependence of the nonlinearity on the distance d_s from soma to branching point was measured for *Branch* stimulation S_{12} ($d_1 = d_2 = 10 \mu\text{m}$). Significant nonlinearity in EPSP summation is apparent. Regardless of the stimulus site distance from the soma, supralinearity was confirmed. The increasing ratios presenting nonlinearity stabilized at less than $100 \mu\text{m}$ distance from soma to branching point. Values are given as mean $\pm$ SE

Fig. 5 Spatiotemporal dependence of EPSP summation in *Line* stimulation. *Line* stimulations (IN: S_{13} , S_{23} and OUT: S_{31} , S_{32}) were applied at different timings ($\tau = 0, 10 \text{ ms}$) and at the same distances ($d_1, d_2, d_3 = 10 \mu\text{m}$). The stimulus sites 1 and 2 for S_1 and S_2 in the *Line* stimulation were decided as such when the superposed EPSP elicited by S_{13} was larger than that by S_{23} . There was significant nonlinearity in the EPSP summation at $\tau = 10 \text{ ms}$, but not 0 ms , where *Line* stimulus S_{31} and S_{32} in the *OUT* direction showed sub-linear EPSP summation. Values are given as mean $\pm$ SE

significant difference between the ratios for S_{13} and S_{23} ($\tau = 0, 10 \text{ ms}$; $d_1, d_2 = 5, 10, 20 \mu\text{m}$), the stimulus sites for S_1 and S_2 were decided by the order ($S_{12} > S_{21}$) of superposed EPSPs at $\tau = 10 \text{ ms}$.

No significant nonlinearity was found for EPSP summations induced by S_{13} and S_{23} in the *IN* direction at either $\tau = 0$ or 10 ms (S_{13} : 1.05 ± 0.03 ($n = 23$); S_{31} : 1.04 ± 0.04 ($n = 23$); S_{23} : 0.97 ± 0.03 ($n = 23$); S_{32} : 1.03 ± 0.04 ($n = 23$)). For stimulations S_{31} and S_{32} in the *OUT* direction, nonlinearity was not significant for

$\tau = 0 \text{ ms}$ but was significant for $\tau = 10 \text{ ms}$. Here, *Line* stimulations S_{31} and S_{32} in the *OUT* direction showed sub-linear EPSP summation (S_{13} : 1.03 ± 0.03 ($n = 22$); S_{31} : 0.89 ± 0.07 ($n = 4$); S_{23} : 1.03 ± 0.03 ($n = 22$); S_{32} : 0.86 ± 0.08 ($n = 4$); t-test, $P < 0.05$).

Results for *Branch* stimulations (S_{12} , S_{21}) applied at different timings ($\tau = 0, 5, 10 \text{ ms}$) and at the same distances ($d_1, d_2 = 5, 10 \mu\text{m}$) are shown in Fig. 6. Stimulus sites 1 and 2 for S_1 and S_2 in the *Branch* stimulation were decided as such when the superposed EPSP elicited by S_{12} was larger than that by S_{21} at $\tau = 5 \text{ ms}$. A significant difference was found between the measured EPSPs for S_{12} and S_{21} (Fig. 6a, $P < 0.05$, $d_1, d_2 = 5 \mu\text{m}$). Significant supralinearity was found for the ratio at $\tau = 0 \text{ ms}$ in both the 5 and $10 \mu\text{m}$ distances, while no nonlinearity was found at $\tau = 5$ and 10 ms (Fig. 6a, b) ($d_1, d_2 = 5 \mu\text{m}$: $\tau = 0 \text{ ms}$, $S_{12,21}$: 1.22 ± 0.05 ($n = 7$); $\tau = 5 \text{ ms}$, $S_{12}(\text{large})$: 1.06 ± 0.08 ($n = 7$), $S_{21}(\text{small})$: 0.84 ± 0.07 ($n = 7$); $\tau = 10 \text{ ms}$, $S_{12}(\text{large})$: 0.99 ± 0.06 ($n = 7$), $S_{21}(\text{small})$: 0.98 ± 0.04 ($n = 7$); $d_1, d_2 = 10 \mu\text{m}$: $\tau = 0 \text{ ms}$, $S_{12,21}$: 1.19 ± 0.03 ($n = 14$); $\tau = 5 \text{ ms}$, $S_{12}(\text{large})$: 1.04 ± 0.04 ($n = 13$), $S_{21}(\text{small})$: 0.97 ± 0.05 ($n = 13$); $\tau = 10 \text{ ms}$, $S_{12}(\text{large})$: 1.03 ± 0.05 ($n = 13$), $S_{21}(\text{small})$: 1.04 ± 0.04 ($n = 13$)). Interestingly, only *Branch* stimulations at $\tau = 5 \text{ ms}$ and $d_1 = d_2 = 5 \mu\text{m}$ showed a significant difference in the ratio between S_{12} and S_{21} large and small measured EPSPs (Fig. 6a) ($P < 0.05$). Since there was no significant nonlinearity in both S_{12} and S_{21} , even though the *Branch* stimulations at $\tau = 5 \text{ ms}$ and $d_1 = d_2 = 5 \mu\text{m}$ showed a significant ratio difference. The results showed that only $\tau = 0 \text{ ms}$ elicited nonlinearity in superposed EPSPs at both 5 and $10 \mu\text{m}$ distances.

Molecular mechanisms of supralinear amplification

To determine the underlying molecular mechanism of supralinear amplification of EPSP summation in the *Branch* stimulation, nonlinearity was measured in the bath application of channel blockers (DL-AP5, Ni^{2+}). Experiments were performed at $d_1 = d_2 = 10$ and $20 \mu\text{m}$ and $\tau = 0 \text{ ms}$. Figure 7a shows the time courses of standardized EPSP (dark gray: measured EPSP, light gray: EPSP linear summation). Under the application of channel blockers, EPSPs were standardized based on the control EPSP linear summation. Figure 7b shows summarized data of the pharmacological effect at $d_1, d_2 = 10 \mu\text{m}$. Upon the addition of DL-AP5 (NMDA receptor blocker) to the ACSF, supralinearity of the measured EPSP was still observed and showed no significant difference compared with the control condition, though the mean peak amplitude showed a small reduction. On the other hand, Ni^{2+} application showed a significant reduction in supralinearity compared with the control condition (ANOVA, $P < 0.05$), and the presence of both Ni^{2+} and DL-AP5 showed an even a

Fig. 6 Spatiotemporal dependence of EPSP summation in *Branch* stimulation. *Branch* stimulations (S_{12} , S_{21}) were applied at different timings ($\tau = 0, 5, 10$ ms) and at the same distances ($d_1, d_2 = 5, 10 \mu m$). The stimulus sites 1 and 2 for S_1 and S_2 in the *Line* stimulation were decided as such when the superposed EPSP elicited

by S_{12} was larger than that by S_{21} at $\tau = 5$ ms and $d_1, d_2 = 5 \mu m$. There was a significant difference between the measured EPSPs for S_{12} and S_{21} . Results show supra-linearity of the ratio only at $\tau = 0$ ms for both 5 and 10 μm distances. There was no nonlinearity at $\tau = 5$ and 10 ms. Values in **a** and **b** are given as mean $\pm$ SE

Fig. 7 Molecular mechanisms of supralinear amplification. The supralinear amplification of EPSP summation in *Branch* stimulations ($d_1, d_2 = 10$ or $20 \mu m$, $\tau = 0$ ms) was measured in bath application of channel blockers (DL-AP5, Ni^{2+}). **a** Standardized EPSP time courses (dark gray measured EPSP, light gray EPSP linear

summation). **b** Significant difference with the control condition at 10 μm distance (ANOVA, $P < 0.05$). There was also greater significant difference in the presence of both Ni^{2+} and DL-AP5 (ANOVA, $P < 0.01$). **c** No significant differences (d_1, d_2) in any of the application conditions. Values in **b** and **c** are given as mean $\pm$ SE

greater significant difference compared with the control condition (ANOVA, $P < 0.01$) ($d_1, d_2 = 10 \mu m$: control: 1.24 ± 0.03 ($n = 47$); AP5: 1.21 ± 0.04 ($n = 8$); Ni^{2+} : 1.04 ± 0.03 ($n = 9$); $Ni^{2+} + AP5$: 1.01 ± 0.02 ($n = 5$)). However, there were no significant differences for $d_1, d_2 = 20 \mu m$ in any of the application conditions ($d_1, d_2 = 20 \mu m$: control: 1.08 ± 0.03 ($n = 26$); AP5: 1.04 ± 0.05 ($n = 5$); Ni^{2+} : 0.91 ± 0.09 ($n = 8$); $Ni^{2+} + AP5$: 0.96 ± 0.05 ($n = 4$)) (Fig. 7c).

Discussion

In this study, to investigate the integration of spatiotemporal inputs on dendrites, two types of pairing stimulations

(*Line* and *Branch*) were applied, using a high-speed laser uncaging system, to dentate GC dendrites.

First, the nonlinearity in EPSP summation induced by coincident application of two inputs was estimated. When the *Line* stimulations were applied with the time interval $\tau = 0$ ms at the same distances from the branching point ($d_1 = d_2 = d_3$; 5, 10, 20 μm), there was no nonlinearity in the input–output relations (the ratios of the peak of measured EPSP and the peak of EPSP linear summation) even with an increase in distance from branching point to stimulation point (Fig. 2). These results indicate that the EPSP summation elicited by subthreshold inputs in the direction from the distal dendrite to the soma along a dendrite was linear, independent of distance from the branching point. This is consistent with the findings of

previous studies using two-photon glutamate uncaging in dentate GCs (Krueppel et al. 2011) and cortical pyramidal cells (Hargreaves et al. 2005; Jackson and Scharfman 1996). On the other hand, when the *Branch* stimulations were applied coincidentally ($\tau = 0$ ms) and at the same distances ($d_1, d_2 = 5, 10, 20, 30 \mu\text{m}$), significant nonlinearity was shown for 5 and 10 μm distances, and the supralinearity gradually decreased with increasing distance (Fig. 3). These results suggest that EPSP integration of inputs within 10 μm of the branching tufts was facilitated if they coincidentally reached the dendritic tufts (daughter branch). The integration of the inputs was large when close to a branching point. Krueppel et al. (2011) showed that EPSP integration by the application of inputs to two dendritic tufts by two-photon uncaging stimulation were linear in GCs. Branco and Häusser (2011) also reported the linearity of EPSP summation in compartmental areas in layer 5 of cortical pyramidal neurons. Both these reports highlight the very important point that dendritic integration performed in compartment segments is linear. However, their stimulation sites were over 20 μm from the branching points, were more sparse, and were further from the branching points than the stimulation sites in our study. Our results indicate that the dendritic integration across a branching point might require both a close distance and a coincidence of inputs for daughter branches. Therefore, nonlinear summation of dendritic EPSP by coincident or oscillated inputs in close proximity to the branching point might facilitate linear summation in dendritic compartment areas. In addition, the dependence of the nonlinearity on the distance d_s between the branching point and soma was measured for the *Branch* stimulation (Fig. 4). Significant nonlinearity in the EPSP summation was observed. The increasing ratios presenting nonlinearity stabilized at 100 μm or less, from soma to branching point. It has been reported that the branching points in GCs are widely distributed from the medial dendrite to the proximal dendrite and sparse at the distal dendrite (Claiborne et al. 1990). Therefore, the results suggest that the boosting of EPSP summation might occur for various information processes, associational–commissural inputs, and inputs from the entorhinal cortex on the middle and distal dendrites. Only a single datum point was obtained at approximately 180 μm due to the thinness of the dendritic tips and limited number of dendritic branches.

Second in this study, pairing stimulations were applied at different timings and different distances to clarify the spatiotemporal dependence of the linearity or nonlinearity in EPSP summation. When the *Line* stimulations were applied, EPSP summations induced in both the *IN* and *OUT* directions at $\tau = 0$ ms showed no nonlinearity (Fig. 5). However, there was a sublinear EPSP summation for the *Line* stimulation in the *OUT* direction at $\tau = 0$ ms. Branco

et al. (2010, Suppl. Fig. 8) reported sensitivity for the sequence of the synaptic activation, in which EPSP summation for the *IN* direction was larger than that for the *OUT* direction in cortical pyramidal cells (Branco et al. 2010). The order of EPSP summation was the same for our results, but unlike our study at $\tau = 0$ ms, their EPSP summation for the *OUT* direction also showed supralinearity. It is considered as the difference that their stimulations were applied more broadly (distance range: $97 \pm 20 \mu\text{m}$ (mean $\pm$ SD)) and more intensely (8–10 pulses) than those of our study (distance range: 10–40 μm , 2 pulses), and their experiments were performed on cortical pyramidal cells, while ours were performed on dentate GCs. The effects of the pairing stimulation in our study were not due to NMDA channels because even EPSP summation for the *IN* direction showed no significant difference in the ratio. Therefore, our stimulation did not elicit a cumulative EPSP induced by continuous NMDA channel activation. On the other hand, Branco et al. (2010) showed supralinearity by NMDA channel activation in both *IN* and *OUT* sequential synaptic activations. Consequently, it is possible that NMDA activation boosted EPSP summation, keeping the order of EPSP summation by *Line* inputs in the *IN* and *OUT* directions.

Next, *Branch* stimulations were applied at different timings (Fig. 6). In the present study, *Branch* stimulations S_{12} and S_{21} were decided as the order of the input–output ratio at $\tau = 5$ ms because there was a significant difference between measured EPSPs for S_{12} and S_{21} (Fig. 6a). This difference might have been caused by the disparity in size of the daughter branches, an effect which is well established (Rall 1962; Kubota et al. 2011). The results showed that only $\tau = 0$ ms elicited nonlinearity in the superposed EPSP at both 5 and 10 μm distances. This suggests that the coincidence is required to integrate inputs for several branches. In other words, inputs to branches were integrated only if they coincidentally reached the branch dendrites.

To clarify the underlying molecular mechanism of the nonlinear summation of EPSP on dendrites, we applied two antagonists to the ACSF. The supralinearity was mainly dependent on the voltage-dependent Ca^{2+} channel and slightly dependent on the NMDA receptors. The results point to a different mechanism of amplitude summation for the direction of multi-inputs along a dendrite, one that is dependent on the NMDA channel (Branco et al. 2010). A possible reason for the nonlinearity in our experiment is the activation of the Ca^{2+} channel since the clamping resting voltage was low (-80 mV). There are Ca^{2+} channels throughout the dendrites in dentate GCs. Moreover, these channels activate at low level potentials (McRory et al. 2001; Aradi and Holmes 1999). It is feasible that Ca^{2+} channels were influenced by the facilitation of EPSPs by simultaneous inputs around the branching point.

GC dendrites differ from those of hippocampal pyramidal cells in morphology and passive electric features (Amaral et al. 2007; Jaffe and Carnevale 1999; London and Häusser 2005; Schmidt-Hieber et al. 2007). In particular, there is strong voltage attenuation in the cells to prevent them from firing due to synaptic input integration (Krueppel et al. 2011). Most synaptic inputs to mossy cells arrive from dentate GCs, and over 90 % of the axon cloud of mossy cells targets GC dendrites at the inner molecular layer (Aradi and Holmes 1999; Buckmaster et al. 1992; Buckmaster et al. 1996). Thus, dentate GCs and hilar mossy cells have recurrent connections whose circuit provides positive feedback and can be considered a form of “recurrent excitation” (Jackson and Scharfman 1996). Moreover, it is thought that the hilar mossy cell circuit controls dentate GC excitability (Jinde et al. 2013). Therefore, the nonlinearity found at proximal dendrites in the present study may serve to support and facilitate the positive feedback from hilar mossy cells, which boosts the integration of inputs to dentate GCs. Inputs to the dendrites of the middle molecular layer originate from layer II of the medial entorhinal cortex through the medial PP (Nishimura-Akiyoshi et al. 2007). The stellate cells, the main cells in the medial entorhinal cortex, generate persistent rhythmic subthreshold voltage oscillations at the theta frequency range (Alonso and Klink 1993; Tahvildari and Alonso 2005). Therefore, the nonlinearity induced by coincident branch inputs to middle dendrites in our study may facilitate the integration of oscillatory inputs in dendrites with strong attenuation, acting as coincidence detectors. In addition, spatial information is delivered to the middle dendrite (Fyhn et al. 2004; Hayman and Jeffery 2008). In this case, the nonlinearity of input summation around the branching point might be used to enhance spatial information. On the other hand, non-spatial information is delivered to the distal dendrite (Hargreaves et al. 2005; Yoganarasimha et al. 2011), and the nonlinearity of input summation here might facilitate non-spatial information.

The nonlinearity in EPSP summation for the synaptic inputs around a branching point does not have a direct or strong effect on input integration like that of NMDA channel-dependent input facilitation (Branco and Häusser 2011). However, these findings on nonlinearity suggest the importance of facilitation at branching points for information integration and coding in dendritic computation. We believe that this nonlinearity may play a crucial role in the integration of synaptic inputs as a facilitator for positive feedback and as a coincidence detector for oscillatory inputs.

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References

- Alonso A, Klink R (1993) Differential electro responsiveness of stellate and pyramidal-like cells of medial entorhinal cortex layer II. *J Neurophysiol* 70(1):128–143
- Amaral DG, Scharfman HE, Lavenex P (2007) The dentate gyrus: fundamental neuroanatomical organization (dentate gyrus for dummies). *Prog Brain Res* 163:3–22. doi:[10.1016/S0079-6123\(07\)63001-5](https://doi.org/10.1016/S0079-6123(07)63001-5)
- Aradi I, Holmes WR (1999) Role of multiple calcium and calcium-dependent conductances in regulation of hippocampal dentate granule cell excitability. *J Comput Neurosci* 6(3):215–235
- Branco T, Häusser M (2010) The single dendritic branch as a fundamental functional unit in the nervous system. *Curr Opin Neurobiol* 20(4):494–502. doi:[10.1016/j.conb.2010.07.009](https://doi.org/10.1016/j.conb.2010.07.009)
- Branco T, Häusser M (2011) Synaptic integration gradients in single cortical pyramidal cell dendrites. *Neuron* 69(5):885–892. doi:[10.1016/j.neuron.2011.02.006](https://doi.org/10.1016/j.neuron.2011.02.006)
- Branco T, Clark BA, Häusser M (2010) Dendritic discrimination of temporal input sequences in cortical neurons. *Science* 329(5999):1671–1675. doi:[10.1126/science.1189664](https://doi.org/10.1126/science.1189664)
- Buckmaster PS, Strowbridge BW, Kunkel DD, Schmiede DL, Schwartzkroin PA (1992) Mossy cell axonal projections to the dentate gyrus molecular layer in the rat hippocampal slice. *Hippocampus* 2(4):349–362. doi:[10.1002/hipo.450020403](https://doi.org/10.1002/hipo.450020403)
- Buckmaster PS, Wenzel HJ, Kunkel DD, Schwartzkroin PA (1996) Axon arbors and synaptic connections of hippocampal mossy cells in the rat in vivo. *J Comp Neurol* 366(2):271–292
- Claiborne BJ, Amaral DG, Cowan WM (1990) Quantitative, three-dimensional analysis of granule cell dendrites in the rat dentate gyrus. *J Comp Neurol* 302(2):206–219. doi:[10.1002/cne.903020203](https://doi.org/10.1002/cne.903020203)
- Fyhn M, Molden S, Witter MP, Moser EI, Moser MB (2004) Spatial representation in the entorhinal cortex. *Science* 305(5688):1258–1264. doi:[10.1126/science.1099901](https://doi.org/10.1126/science.1099901)
- Hargreaves EL, Rao G, Lee I, Knierim JJ (2005) Major dissociation between medial and lateral entorhinal input to dorsal hippocampus. *Science* 308(5729):1792–1794. doi:[10.1126/science.1110449](https://doi.org/10.1126/science.1110449)
- Hayman RM, Jeffery KJ (2008) How heterogeneous place cell responding arises from homogeneous grids—a contextual gating hypothesis. *Hippocampus* 18(12):1301–1313. doi:[10.1002/hipo.20513](https://doi.org/10.1002/hipo.20513)
- Jackson MB, Scharfman HE (1996) Positive feedback from hilar mossy cells to granule cells in the dentate gyrus revealed by voltage-sensitive dye and microelectrode recording. *J Neurophysiol* 76(1):601–616
- Jaffe DB, Carnevale NT (1999) Passive normalization of synaptic integration influenced by dendritic architecture. *J Neurophysiol* 82(6):3268–3285
- Jinde S, Zsiros V, Nakazawa K (2013) Hilar mossy cell circuitry controlling dentate granule cell excitability. *Front Neural Circuits* 7:14. doi:[10.3389/fncir.2013.00014](https://doi.org/10.3389/fncir.2013.00014)
- Kojima H (2006) Development of a system for patterned rapid photolysis and 2-photon confocal microscopy. *Circuits Devices Mag IEEE* 22:66–74
- Krueppel R, Remy S, Beck H (2011) Dendritic integration in hippocampal dentate granule cells. *Neuron* 71(3):512–528. doi:[10.1016/j.neuron.2011.05.043](https://doi.org/10.1016/j.neuron.2011.05.043)
- Kubota Y, Karube F, Nomura M, Gullledge AT, Mochizuki A, Schertel A, Kawaguchi Y (2011) Conserved properties of dendritic trees in

- four cortical interneuron subtypes. *Sci Rep* 1:89. doi:[10.1038/srep00089](https://doi.org/10.1038/srep00089)
- London M, Häusser M (2005) Dendritic computation. *Annu Rev Neurosci* 28:503–532. doi:[10.1146/annurev.neuro.28.061604.135703](https://doi.org/10.1146/annurev.neuro.28.061604.135703)
- McRory JE, Santi CM, Hamming KS, Mezeyova J, Sutton KG, Baillie DL, Stea A, Snutch TP (2001) Molecular and functional characterization of a family of rat brain T-type calcium channels. *J Biol Chem* 276(6):3999–4011. doi:[10.1074/jbc.M008215200](https://doi.org/10.1074/jbc.M008215200)
- Nishimura-Akiyoshi S, Niimi K, Nakashiba T, Itohara S (2007) Axonal netrin-Gs transneuronally determine lamina-specific subdendritic segments. *Proc Natl Acad Sci USA* 104(37):14801–14806. doi:[10.1073/pnas.0706919104](https://doi.org/10.1073/pnas.0706919104)
- Rall W (1962) Electrophysiology of a dendritic neuron model. *Biophys J* 2(2 Pt 2):145–167
- Schmidt-Hieber C, Jonas P, Bischofberger J (2007) Subthreshold dendritic signal processing and coincidence detection in dentate gyrus granule cells. *J Neurosci Off J Soc Neurosci* 27(31):8430–8441. doi:[10.1523/JNEUROSCI.1787-07.2007](https://doi.org/10.1523/JNEUROSCI.1787-07.2007)
- Tahvildari B, Alonso A (2005) Morphological and electrophysiological properties of lateral entorhinal cortex layers II and III principal neurons. *J Comp Neurol* 491(2):123–140. doi:[10.1002/cne.20706](https://doi.org/10.1002/cne.20706)
- Wang X, Lambert NA (2003) Membrane properties of identified lateral and medial perforant pathway projection neurons. *Neuroscience* 117(2):485–492. doi:[10.1016/s0306-4522\(02\)00659-0](https://doi.org/10.1016/s0306-4522(02)00659-0)
- Yoganarasimha D, Rao G, Knierim JJ (2011) Lateral entorhinal neurons are not spatially selective in cue-rich environments. *Hippocampus* 21(12):1363–1374. doi:[10.1002/hipo.20839](https://doi.org/10.1002/hipo.20839)
- Yoneyama M, Fukushima Y, Tsukada M, Aihara T (2011) Spatio-temporal characteristics of synaptic EPSP summation on the dendritic trees of hippocampal CA1 pyramidal neurons as revealed by laser uncaging stimulation. *Cogn Neurodyn* 5(4):333–342. doi:[10.1007/s11571-011-9158-9](https://doi.org/10.1007/s11571-011-9158-9)