Asymmetric spread of excitatory synaptic potential in hippocampal neuronal dendrites revealed by voltage imaging

Masato Morita¹, Reo Higashi¹, Shin-ya Kawaguchi¹,*

¹Department of Biophysics, Graduate School of Science, Kyoto University, Kitashirakawa Oiwake-cho, Sakyō-ku, Kyoto 606-8502, Japan

*Correspondence

Corresponding author: Shin-ya Kawaguchi,
e-mail: kawaguchi.shinya.7m@kyoto-u.ac.jp
tel: +81-75-753-4200

Keywords
voltage imaging, hippocampal neuron, dendrite, excitatory postsynaptic potential (EPSP), local computation
SUMMARY

Despite intensive studies for decades on synaptic integration in the somatodendritic compartments, its spatio-temporal dynamics remains obscure because of the technical limitation to obtain local potentials at multiple subcellular regions. To address this issue, here we used a genetically-encoded voltage indicator together with local uncaging of glutamate or GABA on cultured hippocampal pyramidal neurons. Fluorescence voltage imaging revealed direction-dependent opposite changes of excitatory postsynaptic potentials (EPSPs) in a dendrite: EPSPs attenuated during propagation toward the soma whereas amplified toward the distal branch end. The direction-dependent local augmentation of EPSPs was mediated by the TTX-resistant Na$^+$ channels and KCC2-dependent deeper dendritic resting potential than in soma, the latter of which was directly unveiled by subcellular patch-clamp recordings from a thin dendrite hundreds $\mu$m away from the soma. Taken all these results together, our data identifies an asymmetric modulation of excitatory signals in neuronal dendrites, potentially impacting local integration in a branch.
INTRODUCTION

Neurons extend elaborate dendrites to receive synaptic inputs from other neurons. These synaptic inputs are spatially and temporally integrated in an analogue manner, and then the signals are transformed into action potentials (APs) as digital all-or-none signals at the axon initial segment. It has been shown that hippocampal and cortical pyramidal neurons exhibit several types of regenerative local spikes upon synaptic inputs in dendrites, including Na⁺, NMDA, and Ca²⁺ spikes. Na⁺ and NMDA spikes are triggered by local multiple synaptic inputs in dendritic branches. On the other hand, Ca²⁺ spikes mediated by voltage-gated Ca²⁺ channels, show larger and broader waveforms, often leading to burst APs. In contrast to such a dendritic supra-linear computation mediated by active conductances, sub-threshold potential changes are thought to obey the passive membrane properties of dendrites, attenuating during the propagation as the cable theory predicts. However, at present, it remains elusive how the subthreshold potential changes are processed in dendrite because of the difficulty to apply the patch-clamp technique to multiple points of a thin dendrite at the same time.

As a corollary, mathematical model of electrical circuit assuming multiple compartments has provided basic view of signal integration in dendrites, which still remains to be consolidated by direct experimental analysis.
In this decade, genetically-encoded voltage indicators (GEVIs), which detect membrane potential changes as fluorescence changes, have drastically developed.\textsuperscript{13,14,15} This technique has enabled to fluorescently detect membrane potential changes at a tiny structure which is difficult to apply the patch-clamp technique, for example even a dendritic spine and a presynaptic bouton.\textsuperscript{16,17} Taking advantage of the leading-edge voltage imaging technique, in this study, we studied how synaptic potentials are processed in dendrites. Using improved version of ASAP1 (Accelerated Sensor of Action Potentials 1),\textsuperscript{13} a fluorescent voltage imaging probe, expressed in a cultured hippocampal neuron in combination with very local glutamate or GABA stimulation on a dendrite by 405 nm spot-laser-mediated photoactivation, we found that glutamatergic PSPs spread in dendrites with opposite modulation depending on the direction of propagation. We also identified a novel Cl\textsuperscript{-}-dependent mechanism underlying the unique asymmetric processing of the electrical signal in dendrites.

RESULTS

Fluorescent imaging of membrane potential changes in a cultured hippocampal neuron
To improve voltage imaging probe ASAP1 for higher sensitivity to membrane potential changes, some of amino acid replacements reported in an improved version ASAP3 were introduced into ASAP1 (here we note this version ASAP3β; for details, see methods). We evaluated fluorescence change of ASAP3β upon voltage changes by expressing in HEK293T cells (Figure S1A). ASAP3β exhibited fluorescence decrease (-40.0 ± 1.1 % ΔF/F, Figures S1B and S1C) in response to 100 mV depolarization of the voltage-clamped HEK293T cell from a holding potential at -70 mV, which corresponded to almost two-fold sensitivity of the original ASAP1 (-18.5 ± 1.2 % ΔF/F, Figure S1C) similarly to ASAP3.

We then examined how ASAP3β detects neuronal activity characterized by rapid membrane potential changes in a cultured hippocampal neuron by expressing it with electroporation (Figure 1A). Both the fluorescence and the membrane potential were simultaneously recorded by imaging and patch-clamp methods. ASAP3β detected spontaneous excitatory postsynaptic potentials (EPSPs) almost perfectly without any noticeable change in time course (Figure 1B), showing ~ 1 % fluorescence change for 2 mV membrane potential change (Figure 1C). On the other hand, more rapid spontaneous or evoked APs were detected as smaller fluorescence changes relative to the actual change of membrane potential (-8.4 ± 0.4 % ΔF/F upon 93.6 ± 2.7 mV, Figures 1B and
1C), likely because of the insufficient velocity of ASAP3β to follow a given rapid voltage change (Figures 1B and 1C). From these observations, it was confirmed that ASAP3β nicely detects synaptic potentials.

Direction-dependent EPSP modulation in dendrites

Taking advantage of real-time fluorescent detection of local membrane potential changes of interest throughout dendritic arborization, we studied how synaptic potentials propagate in dendrites. Glutamatergic EPSPs were evoked by local photo-activation of MNI-caged-glutamate (300 μM in the external bath) by 405 nm laser illumination (2 μm diameter) at a spine of ASAP3β-expressing hippocampal pyramidal neuron in culture (Figure 1D). Simultaneous recordings of membrane potentials by current-clamp and fluorescent imaging from an ASAP3β-expressing neuron showed similar waveforms of EPSPs characterized by ~ 1.2 % fluorescence change at the soma upon 2 mV potential change without temporal lag (Figure 1D). Thus, evoked EPSP (eEPSP) measurement by ASAP3β enabled us to quantify the spatial pattern of EPSP spreading in a neuron.

Using this method, eEPSPs were monitored as the relative ASAP fluorescence changes (ΔF/F) throughout the dendrites of ASAP3β-expressing hippocampal neurons in the view field (Figures 1E and 1F). In line with the prediction of the cable theory, the
fluorescence change for eEPSP ($\Delta F/F_{eEPSP}$) attenuated as propagation toward the soma (Figures 1E and 1G). The $\Delta F/F_{eEPSP}$ decreased by $25 \pm 2\%$ at $33.6 \pm 3.1 \mu m$ away from the uncaged site and by $59 \pm 3\%$ at $97.7 \pm 4.9 \mu m$ (Figure 1H, black circles). Thus, the propagation of EPSPs toward the soma attenuates almost exponentially as characterized by a length constant of $\sim 145 \pm 20 \mu m$. In contrast, surprisingly, EPSPs detected as $\Delta F/F_{eEPSP}$ rather amplified when propagating toward the distal end of a dendritic branch (Figures 1E-1H). The $\Delta F/F_{eEPSP}$ increased by $21 \pm 4\%$ at $54.7 \pm 1.9 \mu m$ away from the uncaged site and by $23 \pm 3\%$ at $110.6 \pm 4.5 \mu m$ (Figure 1H, red), although variable. This is quite contrasting to the classical view of passive traveling of subthreshold membrane potential changes in a cable, in which membrane potential change attenuates during propagation in a dendritic tree regardless of direction. The $\Delta F/F_{eEPSP}$ amplification detected by ASAP imaging during propagation toward the distal end was not dependent on the absolute size of $\Delta F/F_{eEPSP}$ at the uncaged site (ranging from $1.1\%$ to $10.9\%$) that reflects the magnitude of EPSPs (Figures 1I and S1D). Thus, our data indicated that excitatory synaptic inputs are oppositely modulated in a dendrite depending on the direction of propagation.

In our self-customized fluorescent imaging system, the LED illumination is designed to be assembled around the center of view field so that high fluorescent signal is attained
as possible even for a very fast image acquisition. As a result, the absolute fluorescent
signals around the edge of view field tended to be smaller, potentially giving rise to a
tendency for the signal to noise ratio to become lower at those dimmer regions. To test
whether the different intensity of light illumination affects the $\Delta F/F_{eEPSP}$ amplification in
the distal end, we compared $\Delta F/F_{eEPSP}$ signals at identical points of dendrite obtained in
two distinct view fields by moving the microscope. As shown in Figure S2, spatial pattern
of $\Delta F/F_{eEPSP}$ signals in dendrites was little affected by the relative brightness of
fluorescence.

The above results indicated that excitatory synaptic signals amplify during propagation
toward the distal dendrite from the input site. We wondered whether this amplification of
depolarization takes place specifically at the only branch where EPSP was evoked or
broadly occurs also at distal branches irrespective of the location of glutamate uncaging.
To examine this, we analyzed how eEPSP propagates into branches stemming out of
the bifurcation on the way to soma from the uncaged site (Figure S3). Some branches
exhibited $\Delta F/F_{eEPSP}$ augmentation during propagation toward distal dendrites compared
to the signal at the branch points (Figure S3), suggesting that the EPSP amplification
during propagation toward distal dendrites was not necessarily limited to the stimulated
branch. On the other hand, other branches exhibited gradual attenuation of
depolarization even when traveling toward the distal end (Figure S3). These apparently contrasting results imply that not all the branches are equipped with the amplification of depolarization, which would be responsible for the diverse extent of the $\Delta F/F_{EPP}$ amplification even at the stimulated branch as shown in Figure 1H.

**TTX-resistant Na$^+$ channels underlie EPSP amplification in distal dendrites**

To explore the molecular mechanisms of the EPSP amplification in distal dendrites, several ion channels were blocked by pharmacological agents. Voltage-gated Na$^+$ channels (Na$_V$) are known to be present in dendrites of pyramidal neurons,\textsuperscript{19} contributing to backpropagation of spikes.\textsuperscript{20} First, we applied a typical Na$_V$ blocker, tetrodotoxin (TTX, 1 $\mu$M) to the external bath, and examined $\Delta F/F_{EPP}$ propagation by ASAP3$\beta$ imaging (Figure 2A). The EPSP amplification in distal dendrite was not affected by TTX ($\Delta F/F_{EPP}$ amplification, 1.32 ± 0.05, Figures 2A, 2C and 2D). While TTX is a typical Na$_V$ blocker used for inhibition of Na$^+$ spikes, TTX-resistant Na$^+$ channels, such as Na$_V$1.5, 1.8 and 1.9, are also known. Among these, at least Na$_V$1.5 and Na$_V$1.9 are reported to be expressed in hippocampal pyramidal neurons.\textsuperscript{21,22,23} In the presence of lidocaine (5 mM), which broadly inhibits Na$_V$, the $\Delta F/F_{EPP}$ amplification in distal dendrites was not significant ($p = 0.25$, paired t-test, Figures 2B and 2C), although slight augmentation was
still remaining ($\Delta F/F_{eEPSP}$ amplification, $1.12 \pm 0.05$, Figure 2D). Thus, TTX-resistant Na$^+$ channels might partially underlie the EPSP amplification in distal dendrites.

We next examined the possible involvement of other cation channels and glutamate receptors in the EPSP amplification in distal dendrites. Voltage-gated Ca$^{2+}$ channels and NMDA receptors are reported to produce dendritic Ca$^{2+}$ and NMDA spikes upon glutamatergic synaptic inputs, contributing to subcellular local computation by supralinear synaptic integration.$^5,^{24}$ Neither external administration of Cd$^{2+}$ (Ca$^{2+}$ channel blocker, 100 μM, Figure 3A) nor D-AP5 (NMDA receptor antagonist, 100 μM, Figure 3B) affected the $\Delta F/F_{eEPSP}$ amplification during propagation toward distal dendrites, suggesting that Ca$^{2+}$ channels and NMDA receptors are not involved in the EPSP amplification here we are focusing on (Figure 3D). In addition, inhibition of hyperpolarization-activated cation channels (HCN channels) and inward rectifier K$^+$ channels (IRK channels) by application of blockers, ZD7288 and Ba$^{2+}$, respectively, failed to affect the $\Delta F/F_{eEPSP}$ amplification (Figures 3D and S4). Taken all these results together, it is suggested that the positive-feedback activation of TTX-resistant Na$^+$ channels by depolarization may specifically, but partly, contribute to the $\Delta F/F_{eEPSP}$ amplification in distal dendrites.

To confirm the above idea, we next attempted to disturb the positive-feedback
activation of Na\textsubscript{v} by reducing membrane resistance through shunting effect mediated by tonic activation of GABA\textsubscript{A} receptors (GABA\textsubscript{A}Rs). As expected, increased shunting effect by external GABA (50 \textmu M) completely abolished the direction-dependent augmentation of $\Delta F/F_{eEPSP}$ (\(\Delta F/F_{eEPSP}\) amplification, 0.91 ± 0.05; \(p = 0.0016\), Dunnett's test; Figures 3C and 3D). Thus, it is concluded that the $\Delta F/F_{eEPSP}$ amplification depends on the positive-feedback activation of depolarizing system composed of the TTX-resistant Na\textsuperscript{+} channels among voltage-gated conductances.

**GABAergic PSPs attenuate as spread in dendrites irrespective of direction**

To further study the spatial pattern of spreading of membrane potential changes in a dendrite, we examined how GABAergic postsynaptic potentials (PSP\textsubscript{GABA}) spread in dendrites by the ASAP3\beta imaging coupled with local activation of GABA\textsubscript{A}Rs. PSP\textsubscript{GABA} were caused by local uncaging of DPNI-caged-GABA (200-500 \textmu M) with a spot 405 nm laser illumination (Figures 4 and S5), in a similar manner to the local glutamate stimulation. In most cases, local GABA stimulation on a dendrite of hippocampal neuron exhibited little fluorescence change of ASAP3\beta, reflecting the resting membrane potential ($V_{m_{\text{rest}}}$) close to the equilibrium potential for Cl\textsuperscript{-} ($E_{\text{Cl}}$). Some cells exhibited increase of ASAP3\beta fluorescence upon GABA uncaging, corresponding to
hyperpolarization (Figures 4A and S5A). In contrast, only very limited cases showed
depolarization as observed with fluorescence decrease upon the local GABA, which is
probably caused by somehow high internal Cl⁻ ([Cl⁻]ᵢ) (Figures 4B and S5B). In any cases,
PSP\textsubscript{GABA}s were not amplified during the propagation in both directions of dendrites
(Figures 4 and S5), which was a sharp contrast to the asymmetric modulation of
glutamatergic EPSPs (see Figure 1). This discrepancy in the signal processing between
glutamate- and GABA-caused depolarizations made us to assume that the Cl⁻
conductance and/or E\textsubscript{Cl} might play a role in amplification of local depolarization in a distal
dendritic branch.

Low [Cl\textsubscript{i}]\textsubscript{in}-dependent deeper V\textsubscript{m\textsubscript{rest}} in distal dendrites

It is determined by the difference between E\textsubscript{Cl} and local V\textsubscript{m\textsubscript{rest}} whether GABA\textsubscript{A}Rs exert
depolarization or hyperpolarization. Therefore, we attempted to measure V\textsubscript{m\textsubscript{rest}} in
dendrites of hippocampal neurons. For that aim, a direct patch-clamp recording from a
thin dendrite was performed using the EGFP-fluorescence as a guide to precisely target
the pipette (Figures 5A and 5B). Whole-cell current-clamp recording showed that,
surprisingly, the V\textsubscript{m\textsubscript{rest}} was more negative in a dendrite (-75.6 ± 1.5 mV) than in the soma
(-68.3 ± 1.4 mV, p = 0.019, Tukey test, Figure 5C). When the dendritic V\textsubscript{m\textsubscript{rest}} was plotted
against the distance from the soma, there was a tendency for \( V_{m_{\text{rest}}} \) to become more negative in a distal dendrite (-3.5 mV / 100 \( \mu \)m, Figure 5D). In addition to the deeper \( V_{m_{\text{rest}}} \), leak conductance per membrane area size (measured at -70 mV under the voltage-clamp configuration) became higher in a dendrite farther away from the soma (Figure 5E), suggesting that more leak channels are basally open at the distal dendrite compared to the soma.

Taken the above results together, we hypothesized that the deeper \( V_{m_{\text{rest}}} \) in a distal branch might be related to the Cl\(^-\) conductance and/or homeostasis depending on the location. This idea was tested using VU0463271 (10 \( \mu \)M), an inhibitor of KCC2, a type of Cl\(^-\) transporter which predominantly contributes to the low [Cl\(^-\)]\(_{in}\) in mature neurons.\(^{25}\) Immunocytochemistry exhibited more abundant expression of KCC2 in distal dendrites of hippocampal pyramidal neurons than in the soma (1.5-fold, \( p = 0.019 \), Tukey test, Figures 5F and 5G). KCC2 basically establishes low [Cl\(^-\)]\(_{in}\) by excluding Cl\(^-\) out of the cytoplasm, and VU0463271 is expected to bring about higher [Cl\(^-\)]\(_{in}\). When KCC2 was inhibited, the \( V_{m_{\text{rest}}} \) in a dendrite became higher by \( \sim 6 \) mV, while the somatic \( V_{m_{\text{rest}}} \) was not affected, resulting in almost identical potentials at dendrites and soma (dendrite: \(-66.5 \pm 2.0 \text{ mV}, \) soma: \(-67.9 \pm 1.9 \text{ mV}, \) Figures 5C and 5D). On the other hand, the tendency of higher basal leak conductance at the distal dendrite was not affected by the
disruption of low $[\text{Cl}^-]_\text{in}$ (Figure 5E). These results altogether suggested that the lower $[\text{Cl}^-]_\text{in}$ and/or higher $\text{Cl}^-$ conductance in a dendrite than in the soma could be a potential cause for the deeper $V_{\text{m,rest}}$ in dendrites.

**EPSP amplification relies on the low $[\text{Cl}^-]_\text{in}$ in dendrites**

Based on the above results, we considered that the specific $[\text{Cl}^-]_\text{in}$ regulation and/or the resultant deeper $V_{\text{m,rest}}$ in distal dendrites compared with the soma might play a role in the local $\Delta F/F_{\text{eEPSP}}$ amplification. Accordingly, in the presence of VU0463271 (10 $\mu$M), a glutamatergic eEPSP monitored by the ASAP3β imaging tended to exhibit smaller amplification in distal dendrites ($\Delta F/F_{\text{eEPSP}}$ amplification, $1.09 \pm 0.03$; $p = 0.019$, Dunnett’s test, Figures 6A and 6C), implying the involvement of low $[\text{Cl}^-]_\text{in}$ in the dendritic amplification of EPSPs. Moreover, simultaneous inhibition of $\text{Na}^+$ and KCC2 by lidocaine and VU0463271 almost abolished the $\Delta F/F_{\text{eEPSP}}$ amplification ($\Delta F/F_{\text{eEPSP}}$ amplification, $1.04 \pm 0.03$; $p = 0.004$, Dunnett’s test, Figures 6B and 6C). Taken all these data together, it is suggested that the low $[\text{Cl}^-]_\text{in}$ in distal dendrites is a critical factor for the EPSP amplification in distal dendrites together with TTX-resistant $\text{Na}^+$ channels.

**Role of Cl channels in the low $V_{\text{m,rest}}$ and local EPSP amplification**
Finally, we attempted to understand how Cl\textsuperscript{−} contributes to the local augmentation of EPSP spreading in the distal dendrite. Taking the following 2 facts into account: 1), the distal dendritic branch exhibits deeper V\textsubscript{m\textsubscript{rest}} and higher basal leak conductance (see Figure 5); 2), the local augmentation of PSP size is specific to the depolarization mediated by Na\textsuperscript{+} but not by Cl\textsuperscript{−} (see Figures 1 and 4), we speculated that the Cl\textsuperscript{−} conductance at the distal dendrite might be responsible for the higher leak conductance which could bring the V\textsubscript{m\textsubscript{rest}} closer to E\textsubscript{Cl}. Among Cl\textsuperscript{−} channels, hippocampal pyramidal neurons express ClC-2\textsuperscript{26} (Figure 7A), which is hyperpolarization-activated and regulates Cl\textsuperscript{−} homeostasis and membrane excitability\textsuperscript{27,28} Thus, it might be possible that more likely ClC-2 activation by the deeper V\textsubscript{m\textsubscript{rest}} in a distal dendrite would increase the basal Cl\textsuperscript{−} leak conductance, that tends to make the V\textsubscript{m\textsubscript{rest}} closer to the E\textsubscript{Cl} which is kept deep by the powerful action of KCC2 locally (Figure 7B). To test this hypothesis, we constructed a biophysical simple model assuming hyperpolarization-activated Cl\textsuperscript{−} conductance like ClC-2 (see Methods for detail of model). When an excitatory input (brief increase of conductance with a reversal potential at 0 mV) is given, an EPSP-like depolarization is caused, and importantly, the depolarization became larger during the traveling toward a region with enriched hyperpolarization-activated Cl\textsuperscript{−} conductances like ClC-2 (Figures 7C-7E). This local augmentation of depolarization in the model was suppressed either
by the elimination of sensitivity of Cl\textsuperscript{−} conductances to hyperpolarization or by the
reduction of a depolarization-caused Na\textsubscript{V} activation (Figures 7D and 7E). Even when the
synaptic input site is altered, the local EPSP amplification was evident at distal branches
although the extent of augmentation was variable (Figure 7E), in line with the variable
amplification in actual distal branches (see Figure 1). Thus, our simple model supported
the idea that the two voltage-sensitive conductances synergistically contribute to the
local EPSP augmentation at distal branches. Accordingly, ASAP3\(\beta\) imaging experiment
showed that the inhibition of Cl\textsuperscript{−} conductances by NPPB (50 \(\mu\)M), a blocker of relatively
broad types of Cl\textsuperscript{−} channels including ClC-2, applied together with the Na\textsubscript{V} inhibitor
lidocaine strongly suppressed the local augmentation of eEPSP in a distal dendrite
(Figure 7F). Thus, our simulation of EPSP traveling in a cable equipped with 2 positive-
feedback mechanisms, depolarization-activated Na\textsuperscript{+} conductance and
hyperpolarization-activated Cl\textsuperscript{−} conductance, nicely predicted the cooperative action of
those mechanisms to locally augment the excitatory synaptic signals, which was
confirmed by the experiment.

**Direction-dependent spatio-temporal integration of synaptic inputs array**

Neuronal dendrites spatio-temporally integrate inputs at numerous synapses.
Considering our data that glutamatergic synaptic inputs asymmetrically spread in a dendritic tree depending on the direction of propagation, we speculated that the synaptic integration might be affected by the asymmetric modulation of synaptic inputs. Hippocampal pyramidal neurons receive inputs from entorhinal cortex (EC) directly via perforant path (or temporo-ammonic path) at distal apical dendrites, and indirectly via the tri-synaptic circuit (mossy fibers or Schaffer collaterals) at the proximal region. Based on such a circuit organization, in a certain situation, excitatory signals may arrive at an apical dendrite of a single cell with a spatio-temporal context: from distal to proximal, which might have a specific impact on synaptic summation. To test this idea, distinct temporal orders of glutamate photolysis were performed at multiple locations along a branch (10 sites, 35 ms intervals) in the presence of TTX (1 \( \mu \)M). We compared the effects of three different patterns of eEPSP trains: laser spots were illuminated along a branch, (1) in a sequence from the soma toward the end (to distal, a to j, Figure 8A), (2) in a sequence from the end of a branch toward the soma (to soma, j to a, Figure 8A), and (3) at random. Fluorescent imaging of ASAP3\( \beta \) showed that the \( \Delta F/F_{eEPSP} \) at distal region (ROI\(_{\text{distal}}\), Figure 8A) was larger than that either at the uncaged branch or at proximal region (ROI\(_{\text{proximal}}\)) in all three spatio-temporal patterns of stimuli (Figure 8B). Thus, \( \Delta F/F_{eEPSP} \) upon multiple stimuli on a branch is augmented during the propagation
toward the distal end of dendrite, in consistent with the data for single eEPSPs. In addition, the location-dependent $\Delta F/F_{\text{eEPSP}}$ amplification was weakened by application of lidocaine (5 mM) and VU0463271 (10 $\mu$M) (Figure S6A), but not by Cd$^{2+}$ (100 $\mu$M) and D-AP5 (100 $\mu$M) (Figure S6B). Notably, peak of $\Delta F/F_{\text{eEPSP}}$ by 10 uncagings became smaller either by lidocaine & VU0463271 or Cd$^{2+}$ & D-AP5, compared with the control (Figures 8C and S6C), indicating synergistic actions of several positive feedback mechanisms to enhance the multiple excitatory inputs for spatio-temporal summation in dendrites.

Furthermore, pyramidal neuronal dendrites exhibited distinct augmentation of EPSP summation depending on the spatio-temporal order of synaptic activation. Although the $\Delta F/F_{\text{eEPSP}}$ at ROI$_{\text{distal}}$ reached similar peaks in two conditions, “to soma” and “to distal” (Figures 8D and S6D, $p > 0.05$, paired t-test), the signal at a distal branch (ROI$_{\text{distal}}$) steeply summated during glutamate uncagings in a sequence of “to soma” compared to “to distal” (Figure 8D, $p < 0.01$, two-way ANOVA), but not at a proximal branch (ROI$_{\text{proximal}}$). The ratio of the eEPSP train trace (at ROI$_{\text{distal}}$) for “to soma” divided by that for “to distal”, reflecting the preference of “to soma” direction for effective summation of excitatory inputs, was kept higher than 1 during the EPSP array (Figure 8E). Importantly, such an efficient synaptic summation was clearly weakened by lidocaine & VU0463271
(Figure 8D, p > 0.05, two-way ANOVA; and Figure 8E, p < 0.01, two-way ANOVA). Thus, the Na\textsubscript{v}- and low [Cl\textsubscript{i}]-dependent $\Delta F/F_{\text{EPSP}}$ amplification seems to make it possible for a distal branch to distinguish the spatio-temporal pattern of synaptic inputs, resulting in local computation in a distinct manner.

**DISCUSSION**

In this study, using voltage imaging with a genetically-encoded probe ASAP3β combined with local glutamate or GABA uncaging, subcellular patch-clamp recordings, and biophysical model simulation, we explored how glutamatergic and GABAergic synaptic inputs propagated in hippocampal neuronal dendrites. Imaging analysis of membrane potential changes at multiple dendritic branches demonstrated direction-dependent asymmetric propagation of excitatory synaptic inputs in a dendrite, that is, EPSPs attenuated during propagation toward the soma, while amplified toward the distal end. Moreover, the EPSP amplification depended on synergistic actions of TTX-resistant Na\textsuperscript{+} channels and low [Cl\textsubscript{i}]-related Cl\textsuperscript{-} conductance. Thus, the data presented in this study showed asymmetric modulation of glutamatergic synaptic inputs in contrast to the conventional view established from the classical cable theory: subthreshold membrane potential changes gradually attenuate during spreading in dendrites. Together, our
findings provide a novel concept of local modulation of synaptic integration in a single neuron.

**Fluorescent voltage imaging**

To examine the spatio-temporal propagation pattern of synaptic inputs, we used fluorescent voltage imaging with a GEVI. Patch-clamp electrophysiology has been a powerful technique to precisely record electrical signals at a given position in a neuron, although it requires a sophisticated technique. On the other hand, GEVIs enable us to measure membrane potential changes much more easily even from multiple subcellular regions at the same time, that is very difficult to perform by conventional patch-clamp recordings. In this decade, several types of GEVI, including ASAP, have been developed, such as ArcLight, Ace2N-mNeon, Voltron, and so on. Taking advantage of this merit, recent studies applied GEVIs to various preparations in vivo and in vitro, demonstrating neuronal excitability changes dependent on behavioral states in animals. Moreover, GEVIs could be non-invasive compared with whole-cell patch-clamp recordings which is typically accompanied with washout of cytoplasmic components. As demonstrated in this study, [Cl]_\text{in} is critical for the local EPSP amplification (see Figures 5-7). We speculate that monitoring of membrane potential...
without intracellular dialysis must have been essential for the evaluations of such cellular functions. Therefore, the lack of useful technique like voltage imaging based on GEVIs must have veiled the asymmetric EPSP amplification at a distal dendrite.

Modulation of synaptic potentials in dendrites

Previous studies have suggested local augmentation of synaptic inputs by active conductances via voltage-gated cation channels such as Na\(^+\) and/or Ca\(^{2+}\) channels and NMDA receptors.\(^4,6,24,33\) Notably, such augmentation of local excitatory inputs emerges when the input size is relatively large (~5 mV or larger when recorded at the soma). On the other hand, the EPSP amplification in distal dendrites demonstrated here operates even if the amplitude of EPSP is small (estimated to be locally 2 ~ 3 mV). Together, the EPSP amplification mediated by the voltage-dependent Na\(^+\) and Cl\(^-\) conductances demonstrated in this study would complementarily function together with other active conductances which operate in a relatively depolarized situation, to locally enhance membrane potential changes in a relatively wide working range.

Supralinear synaptic integration in the local dendrite dependent on the active conductances has been shown in hippocampal and cortical pyramidal neurons,\(^5,6\) whereas other types of neurons such as dentate gyrus granule cells and cerebellar
interneurons, show linear or sublinear summation.\textsuperscript{34,35,36} Thus, processing of synaptic inputs in dendrites seems diverse in distinct types of neurons. Different thickness and/or branch pattern of dendrites, in addition to different combinations of voltage-gated channels, in distinct types of neurons might define the processing pattern. Hippocampal cultured neuronal preparation used here contains several types of neurons such as pyramidal cells, granule cells and inhibitory interneurons. Among them we here focused on pyramidal cells which clearly exhibited the asymmetric EPSP modulation in dendrites (see Figure 1), while apparent granule cells didn’t show such amplification (not shown). Thus, distinct neuronal types seem equipped with specific machinery for local computation in dendrites.

Asymmetric propagation of EPSPs may play a role in associative establishment of synaptic plasticity such as long-term potentiation or depression. Previous studies demonstrated that EPSPs caused at a dendrite sequentially in the direction toward the soma, likely caused an AP firing in cortical pyramidal neurons.\textsuperscript{37} Furthermore, the long-term plasticity at excitatory synapses tends to be induced by clustered inputs at a dendritic region compared to distributed inputs,\textsuperscript{38} and dendritic spike firing in a distal thin branch is likely to induce the long-term potentiation.\textsuperscript{38,39} Here we demonstrated, as shown in Figure 8, that the asymmetric EPSP modulation mechanism clearly enhanced
the amplitude of EPSPs train at a distal branch depending on the spatio-temporal context of inputs, which potentially raises the probability of cooperative induction of plasticity in a limited dendritic branch. Thus, asymmetric modulation of EPSPs revealed here might associate a given pattern of EPSPs arriving at a distal dendrite, contributing to the local dendritic spikes and long-term plasticity. Hippocampal pyramidal neurons receive inputs from various regions: For example, CA3 pyramidal neurons are innervated from perforating fibers in stratum lacunosum-moleculare, mossy fibers in stratum lucidum, and associational connections in stratum radiatum. The local asymmetric modulation of excitatory synaptic inputs would provide selective summation of such specific synaptic inputs at local area of dendrite.

**Molecular mechanism for the EPSP amplification in distal dendrites**

This study identified a unique mechanism of local augmentation of excitatory synaptic inputs in a distal dendrite: low [Cl\textsuperscript{-}]\textsubscript{i}- and Cl\textsuperscript{-} conductance-dependent enhancement of depolarization in distal dendrites (see Figures 5-7). Our biophysical model simulation indicated that a type of Cl\textsuperscript{-} channel, ClC-2, could constitute a kind of positive-feedback loop to stably keep the membrane potential negative closer to the E\textsubscript{Cl} depending on the channel property of hyperpolarization-mediated opening. In such a situation, a
depolarization could decrease the Cl\(^{-}\) conductance, which indirectly contributes to the
enhancement of propagating depolarizing responses (see Figure 7). Indeed, the direct
subcellular patch-clamp recordings of dendritic branches demonstrated the low [Cl\(^{-}\)\(_{\text{in}}\)]
dependent deeper V\(_{\text{m\_rest}}\) and higher leak conductances at distal branches in a distance-
dependent manner from the soma (see Figure 5). It is intriguing that such a Cl\(^{-}\)-
dependent mechanism to augment the depolarizing responses works together with some
TTX-resistant Na\(^{+}\) channels. In this study, we could not present data directly showing the
specific involvement of CIC-2 channels in the asymmetric EPSP modulation because of
the lack of specific blockers. The direct confirmation of the responsible Cl\(^{-}\) channels is
an important task to be accomplished in a future study.

In addition to the involvement of CIC-2-like Cl\(^{-}\) conductance, distinct E\(_{\text{Cl}}\) may also be
present at distal dendrites contributing to deeper V\(_{\text{m\_rest}}\) there. Previous patch-clamp
recordings suggested that E\(_{\text{Cl}}\) at dendrites may deeper than that at the soma because of
the lower [Cl\(^{-}\)\(_{\text{in}}\)] which is established by KCC2 activity.\(^{42,43,44,45}\) More abundant expression
of KCC2 in spines and vicinity of spines\(^{43,46}\) and/or more efficient Cl\(^{-}\) extrusion by KCC2
might operate at distal dendrites based on the distinct surface/volume ratio, as shown by
Báldi et al.\(^{47}\) in hippocampal pyramidal cells. Furthermore, regulation of KCC2 by
phosphorylation and dephosphorylation in distal dendrites\(^{42,48,49}\) may also give rise to
diverse modes of Cl\textsuperscript{-} homeostasis, potentially impacting the local processing of synaptic potentials in a branch.

Our data demonstrated asymmetric propagation of glutamatergic EPSPs, whereas symmetric propagation of depolarizing PSP\textsubscript{GABA} (see Figures 1 and 4). Depolarizing PSP\textsubscript{GABA} would have been caused by high [Cl\textsuperscript{-}]\textsubscript{in}. In such a case, like in the presence of VU0463271 (see Figure 5), E\textsubscript{Cl} should be higher and the deep V\textsubscript{rest} at a distal dendrite would also be absent, which precludes one of the two critical positive-feedback mechanisms for the EPSP amplification.

In addition to the [Cl\textsuperscript{-}]\textsubscript{in}-mediated dynamic control of membrane potential, TTX-resistant Na\textsuperscript{+} channels were demonstrated to underlie in the local EPSP amplification here. Among three subtypes of TTX-resistant Na\textsuperscript{+} channels, Na\textsubscript{V}1.5, 1.8 and 1.9,\textsuperscript{50} Na\textsubscript{V}1.9 is especially activated at more negative membrane potential (-70 ~ -60 mV).\textsuperscript{51} eEPSPs caused here by spot laser illumination on a spine, was not so large in amplitude: ranging from ~ 1 % to 10 % \(\Delta F/F\), corresponding to from 2 mV depolarization to ~ 20 mV at most. The typical activation of Na\textsubscript{V} is emerging from -40 ~ -50 mV, which would make other subtypes unlikely to be involved in the EPSP amplification. Thus, distinct subtypes of Na\textsubscript{V} with different activation profile might provide neurons an ability to operate in a unique manner to locally compute the voltage signals.
ACKNOWLEDGMENTS

We thank to Drs. M. Midorikawa and T. Inoshita for critical reading of the manuscript and helpful comments. This work was supported by JSPS/MEXT KAKENHI Grant Numbers JP22H02721 and JP22K19360 (SK), the Takeda Science Foundation (SK), the Naito Foundation (SK), and Grant-in-Aid for JSPS Fellows Grant Numbers JP22J14296 and JP22KJ1851 (MM).

AUTHOR CONTRIBUTIONS

MM performed most imaging and patch-clamp experiments and data analysis; RH performed simulation and analysis; SK conceptualized and administrated the study; SK and MM interpreted data and wrote the manuscript. All authors approved the final version.

DECLARATION OF INTERESTS

The authors declare no competing interests.
EXPERIMENTAL MODEL AND SUBJECT DETAILS

HEK293T cells

HEK293T cells were cultured at 37 °C in humidified air containing 5 % CO₂ in DMEM (Nakalai Tesque, Kyoto, Japan) supplemented with 10 % (v/v) FBS (NICHIREI BIOSCIENCES, Tokyo, Japan) and penicillin (100 U/mL)-streptomycin (0.1 mg/mL, Nakalai Tesque). For testing voltage-dependent fluorescence changes, HEK293T cells were transfected with original ASAP1 or modified-ASAP (ASAP3β) using Lipofectamine 3000 (1 µg DNA, 2 µL P3000 reagent, 1.5 µL Lipofectamine for 35 mm dish; Thermo Fisher Scientific, Waltham, MA, USA).

Hippocampal neuron culture and transfection

The animals were maintained and treated according to the NIH guide for the care and use of laboratory animals, and to the ethical guidelines on animal experimentation of Kyoto University. Hippocampi were dissected out from male and female newborn Wistar rats and incubated in Ca²⁺ and Mg²⁺-free Hank’s balanced salt solution (CMF-HBSS) containing 0.1 % trypsin for 5 min at 37 °C. Neurons were dissociated by trituration with a fire-polished Pasteur pipette and seeded on poly-D-lysine (Sigma-Aldrich, St. Louis, MO, USA)-coated glasses in Neurobasal plus medium containing 2 % B27 plus
supplement (Thermo Fisher Scientific) and penicillin (100 U/mL)-streptomycin (0.1 mg/mL, Nakalai Tesque). Some cells were transfected with plasmids by means of electroporation with NEPA21 (Nepa Gene, Chiba, Japan). 1 × 10⁶ cells were mixed with plasmids (6 µg ASAP3β plasmid or 3 µg pCAsalEGFP⁵²) in 100 µL CMF-HBSS. Square electric pulses were applied at 300 V (pulse length, 0.5 ms; two pulses; interval, 50 ms; voltage decay rate, 10 %), followed by additional pulses at 20 V (pulse length, 50 ms; five pulses; interval, 50 ms; voltage decay rate, 40 %; polarity exchanged pulse). Transfected cells were then cultured on the glasses together with the non-treated ones. The cells were cultured at 37 °C in humidified air containing 5 % CO₂. One day after seeding, 75 % of the medium was replaced with fresh one. Since then, half of the medium was replaced every 4 days.

**METHOD DETAILS**

**Plasmid construction**

Plasmids were constructed by standard molecular biology techniques. The coding DNA for ASAP1 was obtained from Addgene (#52519), and some modifications for higher voltage sensitivity (D145Δ, L146G, S147T, N149R, S150G, and H151D) in an improved version ASAP3¹⁸ were introduced by PCR-mediated mutations. Modified-ASAP
(ASAP3β) or original ASAP1 cDNAs was inserted into the vector made from pAAV-MSC by replacement of CMV promotor and beta-globin intron region by CA promotor and intron region from pCAGGS, followed by insertion of WPRE sequence before hGH pA region.

**Electrophysiology**

Whole-cell voltage-clamp recording from an ASAP-expressing HEK293T cell was performed with an amplifier (EPC10; HEKA Elektronik GmbH, Reutlingen, Germany) in an extracellular solution containing (in mM) 145 NaCl, 5 KOH, 2 CaCl₂, 1 MgCl₂, 10 Hepes and 10 glucose (pH 7.3) at room temperature (20-24 °C). Patch pipettes (3-5 MΩ) were filled with an internal solution containing (in mM) 147 CsCl, 5 EGTA, 10 Hepes, 15 CsOH, 2 ATP, 0.2 GTP (pH 7.3). The membrane potential (a holding potential of -70 mV) was changed by step voltage depolarizations and hyperpolarizations to a voltage (-100 mV to 50 mV) for 30 ms.

In recordings from hippocampal neurons in culture for 2-3 weeks, we used an extracellular solution containing (in mM) 120 NaCl, 5 KOH, 2 CaCl₂, 1 MgCl₂, 10 Hepes and 10 glucose (pH 7.3). Patch pipettes (3-5 MΩ, somatic recordings; 20-25 MΩ, dendritic recordings) were filled with an internal solution containing (in mM) 120 K-
gluconate, 7 KCl, 5 EGTA, 10 Hepes, 2 ATP, 0.2 GTP (pH 7.3 adjusted with KOH). In some experiments, TTX (1 μM, FUJIFILM Wako Pure Chemical Corporation, Tokyo, Japan), lidocaine (5 mM, Nakalai Tesque), D-AP5 (100 μM, Tocris Bioscience, Bio-Techne SRL, Minneapolis, MN, USA), Cd²⁺ (100 μM, FUJIFILM Wako Pure Chemical Corporation), ZD7288 (100 μM, Tocris bioscience), Ba²⁺ (30 μM, Nakalai Tesque), VU0463271 (10 μM, Tocris Bioscience) and NPPB (50 μM, Tocris Bioscience) were used to inhibit Na⁺ channels, NMDA receptors, Ca²⁺ channels, HCN channels, IRK channels, KCC2 and Cl⁻ channels, respectively. GABA (50 μM, Nakalai Tesque) was used to activate GABA_A receptors.

Fluorescent imaging

Fluorescence imaging was performed using an inverted microscope IX71 (Olympus, Tokyo, Japan) through a 40× 0.95-NA objective (Olympus) for HEK293T cells, or a 60× 1.42-NA oil-immersion objective (Olympus) for hippocampal neurons. Fluorescent excitation was delivered using a white LED (SOLA; Lumencor, OR, USA) with 15 mW/mm² through a 450-480 nm filter. Fluorescent images were obtained with a Zyla4.2 sCMOS camera (Andor Technology Ltd, Belfast, UK) at 1500 Hz (128 × 128 pixels, binning 2 × 2) for HEK293T cells, at 400 Hz (512 × 512 pixels, binning 2 × 2) or at 200
Hz (1392 × 1040 pixels, binning 2 × 2) for hippocampal neurons and analyzed with SOLIS (Andor) or Image J software (NIH, Bethesda, MD, USA).

Glutamate or GABA uncaging

For glutamate or GABA uncaging, MNI-caged-L-glutamate (300 μM, Tocris Bioscience) or DNPI-caged-GABA (200-500 μM, Tocris Bioscience) was applied to the external solution. Uncaging was caused by 405 nm laser illumination (Single line laser; Rapp OptoElectronic GmbH, Wedel, Germany) with 4-6 mW laser power.\textsuperscript{53,54} Glutamate uncaging was performed by laser illumination at a 2 μm diameter spot (0.2 ms duration; or 1 ms in the presence of external GABA), while GABA was uncaged by a spot or a line scan (0.5-5 ms duration). The location of laser illumination was controlled by a localized photomanipulation system based on Galvano mirrors (UGA-42 Firefly; Rapp OptoElectronic) implemented in SysCon software (Rapp OptoElectronic).

Immunocytochemistry

Cultured neurons were fixed with 4 % paraformaldehyde in phosphate buffered saline, permeabilized with 0.5 % Tween 20, then blocked with 1 % goat serum, and finally labeled with primary and secondary antibodies. The following antibodies were used:
rabbit polyclonal antibodies (pAb) against KCC2 (1:1000, Sigma-Aldrich), ClC-2 (1:5000, Alomone Labs, Jerusalem, Israel), chicken pAb against GFP (1:2000, Sigma-Aldrich), Alexa 568-conjugated pAb against rabbit IgG (1:400, Thermo Fisher Scientific) and Alexa 488-conjugated pAb against chicken IgG (1:400, Thermo Fisher Scientific). Fluorescent images were recorded with a confocal laser microscope (FV1000 imaging system, Olympus, Japan), and analyzed using ImageJ software (NIH). The neuronal dendrites were defined as the area positive for GFP.

Simulation of simple dendrite model with voltage-dependent Na⁺ and Cl⁻ channels

Simple model of a dendrite equipped with the depolarization-activated Na⁺ and the hyperpolarization-activated Cl⁻ channels was constructed and simulated using the NEURON simulator version 8.2 operating under the Python programming language. Here we assumed a multi-compartment model consisting of a soma and 18 dendritic sections with single branch point where 3 daughter branches stemmed out (see Figure 7C), one of which had 120 μm and the other two 90 μm in length, yielding total arborization length of 500 or 470 μm long. The diameter of a dendritic branch was assumed to be 2.5 μm at the most proximal one, narrowing down towards the distal end until it reaches 0.5 μm at 200 μm away from the soma. Cytosolic resistivity was here set
at 250 Ω·cm, and membrane capacitance 0.8 μF/cm². Membrane of individual compartments were equipped with TTX-resistant voltage-gated Na⁺ channel \( \text{Na}_V1.9 \), hyperpolarization-activated Cl⁻ channel like CIC-2, Na⁺/K⁺ pump, KCC2, and linear leak conductances for Na⁺, K⁺, and Cl⁻ (for detailed parameters of each components, see Table S1).

Individual components in each compartment at the membrane potential \( V_m \) were expressed in the model based on the equilibrium potentials for Na⁺ \( E_{Na} \), K⁺ \( E_K \), and Cl⁻ \( E_{Cl} \) by implementing the equations which were already published in previous studies, as described below.

For TTX-resistant persistent Na channels, \( \text{Na}_V1.9 \) model\(^5^6\) was here used:

\[
I_{Na_v} = \overline{g_{Na_v}} \times m_{Na_v} \times h_{Na_v} \times (V_m - E_{Na})
\]

where \( \overline{g_{Na_v}} \), \( m_{Na_v} \), \( h_{Na_v} \) are the maximal conductance, voltage-dependent activation and inactivation probability factors, respectively. Voltage-dependent changes of \( m_{Na_v} \) and \( h_{Na_v} \) were defined by the rate constants \( (\alpha \text{ and } \beta) \) as described by the following equations:

\[
\frac{dm_{Na_v}}{dt} = \alpha m_{Na_v} \times (1 - m_{Na_v}) - \beta m_{Na_v} \times m_{Na_v}
\]

\[
\frac{dh_{Na_v}}{dt} = \alpha h_{Na_v} \times (1 - h_{Na_v}) - \beta h_{Na_v} \times h_{Na_v}
\]

where forward \( (\alpha) \) and reverse \( (\beta) \) rate constants for \( m_{Na_v} \) and \( h_{Na_v} \) were assumed as
below:

\[
\alpha_{m_{Nav}} = \frac{1.032}{1 + \exp \left( \frac{V_m + 6.99}{-14.87115} \right)}
\]

\[
\beta_{m_{Nav}} = \frac{5.79}{1 + \exp \left( \frac{V_m + 130.4}{22.9} \right)}
\]

\[
\alpha_{h_{Nav}} = \frac{0.06435}{1 + \exp \left( \frac{V_m + 73.26415}{3.71928} \right)}
\]

\[
\beta_{h_{Nav}} = \frac{0.13496}{1 + \exp \left( \frac{V_m + 10.27853}{-9.09334} \right)}
\]

For hyperpolarization-activated Cl\(^{-}\) component, a modified biophysical model for ClC-2\(^{57}\) was incorporated:

\[
I_{Cl} = g_{\bar{Cl}} \times m_{Cl} \times (V_m - E_{Cl})
\]

\[
\frac{dm_{Cl}}{dt} = 0.1 \times (m_{Cl,ss} - m_{Cl})
\]

, where \(g_{\bar{Cl}}\) is the maximal conductance, \(m_{Cl}\) is the relative open probability factor of the channel, and its steady state at \(V_m\), \(m_{Cl,ss}\) is defined as:

\[
m_{Cl,ss} = \frac{1}{1 + \exp \left( \frac{V_m - E_{Cl} + V_h}{V_s} \right)}
\]

, where \(V_h\) and \(V_s\) are parameters to define the voltage-sensitivity of the channel activation.

For KCC2, \(K^+\) and Cl\(^{-}\) fluxes were calculated as a product of the parameter for its anti-
porting efficiency ($U_{KCC2}$) and the difference between their equilibrium potentials as demonstrated in Gentiletti et al.\textsuperscript{58}:

$$I_{K(KCC2)} = U_{KCC2} \ln \left( \frac{[K^+]_{in} [Cl^-]_{in}}{[K^+]_{out} [Cl^-]_{out}} \right)$$

$$I_{C(KCC2)} = -I_K$$

Na\textsuperscript{+}/K\textsuperscript{+} pump activity was calculated based on the formulations in Takeuchi et al.,\textsuperscript{59} as listed below,

$$I_{NaK} = I_{NaK_{max}} \big( k_1 \times p_{E1Na} \times y - 0.04 \times p_{E2Na} \times (1 - y) \big)$$

$$I_{K(pump)} = -2I_{NaK}$$

$$I_{Na(pump)} = 3I_{NaK}$$

State transitions of the pump were defined by rate constants as below:

$$\frac{dy}{dt} = \alpha_y (1 - y) - \beta_y y$$

$$\alpha_y = 0.04 \times p_{E2Na} + 0.165 \times p_{E2K}$$

$$\beta_y = \frac{0.37}{1 + \left( \frac{0.094}{[ATP]_{in}} \right) \times p_{E1Na} + 0.01 \times p_{E1K}}$$

$$[Na^+]_{eff} = [Na^+]_{out} \exp \left( -\frac{0.82 \times 96.4853 \times V_m}{8.314463 \times T} \right)$$

$$p_{E1Na} = \left( 1 + \left( \frac{4.05}{[Na^+]_{in}} \right)^{1.06} \left( 1 + \frac{[K^+]_{in}}{32.88} \right)^{1.12} \right)^{-1}$$

$$p_{E1K} = \left( 1 + \left( \frac{32.88}{[K^+]_{in}} \right)^{1.12} \left( 1 + \frac{[Na^+]_{in}}{4.05} \right)^{1.06} \right)^{-1}$$

$$p_{E2Na} = \left( 1 + \left( \frac{69.8}{[Na^+]_{eff}} \right)^{1.06} \left( 1 + \frac{[K^+]_{out}}{0.258} \right)^{1.12} \right)^{-1}$$
36

\[ p_{E_{K}} = \left( 1 + \left( \frac{0.258}{[K^+]_{out}} \right)^{1.12} \left( 1 + \frac{[Na^+]_{eff}}{69.8} \right)^{1.06} \right)^{-1} \]

624 In our model, calculation of the Na\(^+\)/K\(^+\) pump activity was specifically implemented in Neuron simulator, by modifying the program code presented in Botta et al.\(^60\)

627 Notably, here we assumed the hyperpolarization-activated Cl\(^-\) channels only at distal compartments (see Figure 7C). An EPSP was represented as a double exponential conductance change (reversal potential at 0 mV) at a single compartment (#6-8 in Figure 7C), defined by the rise and decay time constants of 10 and 80 ms, respectively, and the peak conductance was optimized to cause similar size of EPSP (~12 mV) at the stimulated compartment. The model was simulated in Neuron platform with backward Euler method (dt = 0.025 ms). In some simulations to evaluate the role of the voltage-dependent Na\(_V\) and Cl\(^-\) conductances in the model, the maximal conductance of Na\(_V\) was halved and/or the Cl\(^-\) conductances at distal branches were set constant.

638 QUANTIFICATION AND STATISTICAL ANALYSIS

639 Data analysis of ASAP fluorescence change

640 Dendritic regions were selected for analysis as ROIs in ImageJ, and \(F_{dend}\) was calculated
as the mean intensity of fluorescence in each ROI which was subtracted by background noise defined as the mean of fluorescence intensity at the area nearby each ROI. During image acquisition of ASAP-expressing cells, fluorescence gradually decreased due to photobleaching, which was defined as \( F_{base} \) obtained by double exponential curve fitting of the total fluorescence change as follows:

\[
F_{base} = A + B \cdot \exp \left\{ \alpha \cdot \left( \frac{-t}{\tau_1} \right) + (1 - \alpha) \cdot \left( \frac{-t}{\tau_2} \right) \right\}
\]

All above parameters (A, B, \( \alpha \), \( \tau_1 \) and \( \tau_2 \)) were determined by the least square method from the time course of fluorescence change before the uncaging. The relative change of fluorescence (\( \Delta F/F \), %) was

\[
\Delta F/F = \left( \frac{F_{dend}}{F_{base}} - 1 \right) \cdot 100
\]

In this study, relative fluorescence change of ASAP is represented as \(-\Delta F/F\) because of the negative relation of fluorescence against membrane potential change. \(-\Delta F/F\) traces in individual ROIs were obtained by averages of 3-7 trials. Adjacent area to the laser spot was excluded from analysis because of an artifact by laser illumination. Distance between two sites of interest was measured using ImageJ (NIH).

**Statistics analysis**

Statistical significance of differences between groups was tested by paired Student's t-
test, Dunnett’s test, Tukey test or two-way ANOVA, and \( P < 0.05 \) was considered as significant. Throughout the project, data are presented as mean \( \pm \) standard error of the mean (SEM) unless otherwise stated. In all figures, symbols with error bars indicate mean \( \pm \) SEM; asterisk, \( P < 0.05 \); double asterisk, \( P < 0.01 \); triple asterisk, \( P < 0.001 \). These analyses were performed by Microsoft Excel and Igor Pro8 (WaveMetrics, Lake Oswego, OR, USA).
Reference


Figure 1. Direction-dependent EPSP modulation in dendrites by voltage imaging

(A) Fluorescent image of a cultured hippocampal neuron expressing ASAP3β under the whole-cell patch clamp recording.

(B) Representative spontaneous EPSP (left) and action potential (right) in a cultured hippocampal neuron, recorded by ASAP imaging (black) and current-clamp recording (red).

(C) Relative fluorescence changes (ΔF/F) for EPSPs (red, 29 events, 5 cells) and action potentials (blue, 12 events, 3 cells) plotted against the size of membrane potential changes (ΔVm).

(D) Top, single-photon (405 nm) glutamate uncaging around a spine of an ASAP3β-expressing neuron under the whole-cell patch-clamp recording. Bottom, somatic voltage change upon glutamate uncaging recorded by current-clamp (red) or fluorescence imaging (black).

(E) Top, image of an ASAP3β expressing neuron. Yellow rectangular area is expanded below, showing branches used for voltage imaging of local glutamate-evoked EPSPs. Magenta point indicates the location of 405 nm spot laser illumination.

(F) ΔF/F_{eEPSP} traces at six ROIs (1-6) indicated in E.

(G) Top, Schematic drawing of classification of dendrites (black, dendrite on the way to soma; red, distal branches; blue, bifurcated branches on the way to soma). Bottom, ΔF/F_{eEPSP} sizes for individual eEPSPs (normalized to that at ROI 3) in a cell (show in E) are plotted against the distance from the uncaged site.

(H) Relative ΔF/F_{eEPSP} against the distance from the uncaged site (21 cells, red: 102, black: 85, blue: 226 regions based on the categorization shown in G). Square plots indicate averages (± SEM).

(I) Absolute ΔF/F_{eEPSP} sizes at three categories of location: distal branch, uncaged site, and proximal branch (n = 21 cells, **: p < 0.01, ***: p < 0.001, paired t-test). Square plots indicate averages (± SEM). See also Figures S1-S3.
Figure 2. Partial contribution of TTX-resistant Na⁺ channels to EPSP amplification

(A) and (B) Left, dendritic segment of interest measured in the presence of TTX (1 μM, A) or lidocaine (5 mM, B). Magenta points indicate the locations of glutamate uncaging. Middle, ΔF/ΔFₑEPSP traces at five ROIs (1-5) indicated in the left. Right, relative ΔF/ΔFₑEPSP plotted against the distance from the uncaged site (20 cells, red: 103, black: 93 regions for A; 17 cells, red: 53, black: 53 regions for B). Square plots indicate averages (± SEM).

(C) Absolute ΔF/ΔFₑEPSP sizes in distal region of dendrites and uncaged sites in the presence of TTX (left, n = 20 cells, *: p < 0.05, paired t-test) or lidocaine (right, n = 17 cells, n.s.: not significant, paired t-test). Square plots indicate averages (± SEM).

(D) ΔF/ΔFₑEPSP amplification in distal region with or without TTX and lidocaine (n.s.: not significant, Dunnett’s test). Error bars are the SEM.
**Figure 3. Abolishment of EPSP amplification by GABA-mediated membrane shunting, but not by blocking types of cation channel**

(A-C) Left, stimulated dendritic segment in the presence of Cd\(^{2+}\) (A, 100 μM), D-AP5 (B, 100 μM) or GABA (C, 50 μM). Magenta points indicate the locations of glutamate uncaging. Middle, \(\Delta F/F_{\text{EPSP}}\) traces at five ROIs (1-5) indicated in left. Right, relative \(\Delta F/F_{\text{EPSP}}\) plotted against the distance from the uncaged site (Cd\(^{2+}\), 15 cells, red: 37, black: 50 regions; D-AP5, 14 cells, red: 42, black: 45 regions; GABA, 10 cells, red: 29, black: 31 regions). Square plots indicate averages (± SEM).

(D) Statistical summary of \(\Delta F/F_{\text{EPSP}}\) amplification in distal branches with or without GABA (50 μM), Cd\(^{2+}\) (100 μM), D-AP5 (100 μM), ZD7288 (100 μM) or Ba\(^{2+}\) (30 μM). Dunnett’s test, ***: p < 0.001, n.s.: not significant. Error bars are the SEM. See also Figure S4.
Figure 4. Symmetric attenuative propagation of GABAergic PSPs

(A) and (B) Left, dendritic segments of interest used for voltage imaging upon local GABA-evoked hyperpolarizing (A) or depolarizing (B) postsynaptic potentials (PSP_{GABA}). Magenta point or line indicates the location of GABA uncaging. Middle, \( \Delta F/F \) for PSP_{GABA} traces at five ROIs (4-8 for A, 2-6 for B) indicated in the left image. Right, relative \( \Delta F/F \) for PSP_{GABA} is plotted against the distance from the uncaged site (6 cells, red: 25, black: 18 regions for A, 8 cells, red: 33, black: 20 regions for B). Square plots indicate averages (\( \pm \) SEM). See also Figure S5.
Figure 5. $[\text{Cl}]_\text{in}$-dependent more negative membrane potential in dendrites

(A) Image of direct patch-clamp recording from an EGFP-expressing dendrite (right) and magnified view of the patched branch in yellow (left).

(B) $V_m$ trace recorded from a dendritic branch (shown in A).

(C) Somatic (blue, $n = 19$ cells) and dendritic (green, $n = 9$ cells) $V_{m\text{rest}}$ without or with $10 \mu\text{M VU0463271}$ (soma: yellow, $n = 9$ cells; dendrite: red, $n = 11$ cells). Tukey test, $^*: p < 0.05$, $^{**}: p < 0.01$, n.s.: not significant.

(D) and (E) $V_{m\text{rest}}$ (D) and leak conductance (E) plotted against the distance from soma.

(VU: in the presence of VU0463271.

(F) Images of immunostained KCC2 (magenta, top) in a GFP-labeled hippocampal neuron (green, middle). Three rectangular areas (a - c) are expanded below with color-merged.

(G) Ratio of fluorescent intensity of KCC2 to GFP in soma, proximal and distal dendrites ($n = 9$ cells, $^*: p < 0.05$, n.s.: not significant, Tukey test). Square plots indicate averages ($\pm$ SEM).
Figure 6. Low $[\text{Cl}]_o$ are involved in EPSP amplification at a dendrite

(A) and (B) Left, stimulated dendritic segment in the presence of VU0463271 (10 $\mu$M, A), or lidocaine (5 mM) and VU0463271 (10 $\mu$M, B). Magenta points indicate the locations of glutamate uncaging. Middle, $\Delta F/F_{\text{EPSP}}$ traces at five ROIs (1-5) indicated in the left image. Right, relative $\Delta F/F_{\text{EPSP}}$ plotted against the distance from the uncaged site (17 cells, red: 44, black: 52 regions for A; 15 cells, red: 38, black: 44 regions for B). Square plots indicate averages ($\pm$ SEM).

(C) Statistical summary of $\Delta F/F_{\text{EPSP}}$ amplification in distal region with or without VU0463271 and lidocaine (*: $p < 0.05$, **: $p < 0.01$, Dunnett’s test). Error bars are the SEM.
Figure 7. Role of Cl^- channels in the low V_{m,rest} and local EPSP amplification

(A) Immunostained CIC-2 image in a hippocampal neuron.

(B) Hypothetical model for mechanisms of EPSP amplification in distal dendrites.

(C) Geometric design of a multi-compartment model equipped with the deep V_{m,rest} activated Cl^- conductance and the low-threshold Na_v channels. Dendrites are composed of 18 compartments, and an excitatory synaptic input was given at the compartment #6, #7, or #8. See also Table S1.

(D) Simulated EPSP traces (caused at #7) at five representative compartments (#1,4,7,9,12) indicated in C, with or without the 50 % inhibition of Na_v and/or the abolishment of deeper V_{m,rest}-activated Cl^- conductance change.

(E) Relative amplitude of simulated EPSPs caused at #6 (right), #7 (middle), or #8 (left) plotted against the distance from the input site in 4 conditions of the model.

(F) Left, ASAP3β-imaged dendritic segment in the presence of lidocaine (5 mM) and NPPB (50 μM). Magenta point indicates the location of glutamate uncaging. Middle, ΔF/ΔF_{0EPSP} traces at five ROIs (1-5) indicated in the left image. Right, relative ΔF/ΔF_{0EPSP} plotted against the distance from the uncaged site (13 cells, red: 37, black: 34 regions). Square plots indicate averages (± SEM).
**Figure 8. Dynamic spatio-temporal integration of synaptic inputs**

(A) Left, image of an ASAP3β-expressing neuron, with yellow rectangular area expanded in right, showing 10 sites of local glutamate uncaging (magenta, 35 ms intervals) in three patterns: to distal (a to j), to soma (j to a) or random.

(B) Averaged $\Delta F/F_{eEPSP}$ traces ($\pm$ SEM, 17 cells) at three ROIs (uncaged branch: black, proximal: blue, distal: red as indicated in A right) upon three patterns of glutamate uncagings. Magenta lines indicate spot laser illumination.

(C) $\Delta F/F_{eEPSP}$ traces (mean $\pm$ SEM) at ROI$_{distal}$ in three conditions: control (red, 17 cells), with lidocaine (5 mM) and VU0463271 (10 μM) (yellow, 13 cells), and with Cd$^{2+}$ (100 μM) and D-AP5 (100 μM) (green, 14 cells).

(D) Comparison of synaptic integration at ROI$_{distal}$ upon three patterns of glutamate inputs in the absence (ctrl, top) or presence of lidocaine and VU0463271 (bottom, **: p < 0.01, ***: p < 0.001, n.s.: not significant, two-way ANOVA).

(E) Ratio of $\Delta F/F_{eEPSP}$ signals (mean $\pm$ SEM) upon opposite directions of glutamate uncagings (“to soma” relative to “to distal”) at ROI$_{distal}$ in the absence (ctrl) or presence of lidocaine and VU0463271. **: p < 0.01, two-way ANOVA. See also Figure S6.