

Synaptic input architecture of visual cortical neurons revealed by large-scale synapse imaging without backpropagating action potentials

Reviewed Preprint

v1 • January 6, 2025

Not revised

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eLife Assessment

This **valuable** study uses a novel method to record spine calcium responses without the confounds of backpropagating action potentials to study how the dendritic integration of large numbers of inputs generates the tuned output of cortical neurons. While the results are generally **solid**, the study would benefit from more details, characterizations, and quantifications, including better validation of the method to suppress backpropagating action potentials.

<https://doi.org/10.7554/eLife.104334.1.sa2>

Abstract

How neurons integrate thousands of synaptic inputs to compute sharply tuned outputs is a critical question in sensory information processing. To answer this question, it is essential to record the location and activity of synaptic inputs *in vivo*. However, back-propagating action potential (BAP) calcium signals invade dendrites and spines, making accurate recording of spine responses difficult. In this study, we first developed a new method to record spine calcium responses without BAP signals. Using this method, we performed large-scale imaging of visually evoked spine activity from layer 2/3 pyramidal neurons and revealed three patterns of dendritic functional architectures of synaptic inputs: dendrites with clusters of spines of similar responses, dendrites with spines of diverse responses, and dendrites with spines where the majority of them show no visual response. Our model suggests that only a small fraction of spines on dendrites of clustered architectures are sufficient to generate sharply tuned output.

Introduction

A single neuron in the central nervous system receives thousands of synaptic inputs and integrates them to generate an action potential (Stuart & Spruston, 2015 [↗](#)). Because the depolarization induced by each individual synapse is relatively weak (Mitchell et al., 2019 [↗](#)), the mechanism by which a single neuron determines its specific output from multiple inputs is not well understood. Over the past few decades, the morphology and electrical properties of dendrites have been studied intensively (Stuart & Spruston, 2019). Clustered synaptic activity in dendrites could lead to enhanced dendritic responses, potentially important for dendritic computation, was first proposed by a modeling study (Mel, 1992 [↗](#)). Later studies have extensively explored this proposal through computer simulations (Hausser & Mel, 2003) and experimental methods by electrophysiology or imaging (Magee, 2000 [↗](#); Branco & Hausser, 2010). Furthermore, the somatic action potentials occur when the membrane potential depolarized by the sum of synaptic activities is only above the threshold (Golding & Spruston, 1998 [↗](#)). Thus, these studies suggest the importance of both simultaneous clustered synaptic activation and the generation of above-threshold somatic action potentials for output signal generation. Because electrophysiological recordings of dendrites do not have sufficient spatial resolution for individual synaptic inputs, it is essential to record both the location and activity of individual synaptic inputs along dendrites using other techniques to answer the question of how neurons integrate synaptic inputs and determine output signals.

Recent technical advances in optical imaging, in particular two-photon calcium imaging, have made it possible to record brain activity in living animals with high spatial and temporal resolution (Helmchen & Denk, 2005 [↗](#)). Using this technique, a large number of synaptic input signals distributed over dendrites can be measured with a high spatial resolution (Jia et al., 2010 [↗](#); Chen et al., 2013 [↗](#); Grienberger et al., 2015 [↗](#)). In the case of pyramidal neurons, excitatory synaptic inputs mainly occur at small dendritic protrusions called spines (Harris & Kater, 1994 [↗](#); Knott et al., 2006 [↗](#); Iascone et al., 2020 [↗](#)). Activation of a single spine induces an increase in calcium transients mediated by N-methyl-D-aspartate (NMDA) receptors, voltage-gated calcium channels or internal calcium stores (Grienberger & Konnerth, 2012 [↗](#); Rochefort & Konnerth, 2012 [↗](#)) and this activity can be measured by calcium indicators (Jia et al., 2010 [↗](#); Chen et al., 2011 [↗](#); Chen et al., 2013 [↗](#)). Using two-photon calcium imaging, the input-output transformation principle of a single neuron has recently been investigated, particularly with regard to neuronal response selectivity in V1 neurons (Chen et al., 2013 [↗](#); Wilson et al., 2016 [↗](#); Iacaruso et al., 2017 [↗](#); Scholl et al., 2021 [↗](#)). The computation of neuronal response selectivity in V1 may provide a general model for understanding the input-output transformation principle of cortical neurons.

A prominent feature of V1 excitatory neurons is orientation selectivity, characterized by selective responses to a particular orientation of lines (Hubel & Wiesel, 1962 [↗](#)). In higher mammals such as monkeys, cats and ferrets with functional columns, these orientation-selective neurons are arranged in a columnar structure, called orientation column. Animals with such functional column, a single excitatory neuron receives relatively uniform inputs from other excitatory neurons belonging to the same orientation-preference column and the mechanism to generate sharp outputs from uniform inputs has been explained experimentally in ferrets (Wilson et al., 2016 [↗](#)). By contrast, in mice without functional columns (salt-and-pepper structure; Ohki et al., 2005 [↗](#); Ringach et al., 2016 [↗](#); Kondo et al., 2016 [↗](#)), a single excitatory neuron generally receives diverse inputs from surrounding excitatory neurons with diverse responses. The predicted output signal from the simple summation of total inputs has much broader tuning than the soma (Chen et al., 2013 [↗](#); Scholl et al., 2021 [↗](#)), and the mechanism that generates the sharp tuning of the soma remains unknown.

Several mechanisms for the generation of sharply tuned outputs have been previously reported. In ferrets, Wilson et al. suggested a contribution of dendritic nonlinearity for the sharpening of the tuning elicited by clustered synaptic activation (Wilson et al., 2016 [DOI](#)). This sharpening mechanism is supported by a study showing that visually evoked NMDA spikes on dendrites enhanced soma orientation selectivity in mice (Smith et al., 2013 [DOI](#)). Another possible mechanism for sharpening is thresholding at the soma. Somatic spiking is regulated by membrane potential thresholding, which allows firing in response to the preferred orientation and prevents firing in response to a non-preferred orientation (Priebe & Ferster, 2008 [DOI](#)). Electrophysiological recordings from cat V1 neurons showed that the orientation tuning of spike responses was narrower than the tuning of the membrane potential (Carandini & Ferster, 2000 [DOI](#)). In contrast, simultaneous *in vivo* spine imaging and electrophysiological recording of soma membrane potential showed a highly variable relationship between spine activity and somatic firing (Wilson et al., 2016 [DOI](#)).

To elucidate the mechanisms to generate the sharp tuning in mice, we first developed a novel method that solves the critical problem in *in vivo* spine imaging: the intrusion of back propagating action potential (BAP) calcium signals into the spine, which potentially leads to inaccurate measurements of the spine calcium signal. The mathematical method has been used to isolate the spine calcium signal by subtracting the dendritic calcium signal (Chen et al., 2013 [DOI](#); Wilson et al., 2016 [DOI](#); Iacaruso et al., 2017 [DOI](#); Scholl et al., 2021 [DOI](#)) but the question remains how accurately this method estimates true spine activity.

In this study, we established a new optogenetic method for imaging spine activity without BAPs using a bistable inhibitory opsin containing a soma-targeting signal. This method has a major advantage over previously reported BAP-inhibition methods (Jia et al., 2010 [DOI](#); Levy et al., 2012 [DOI](#)) in that the prevention of somatic depolarization by photoinhibition is technically easier and lasts for longer, and BAP-free imaging can be performed during this period. By expressing a bistable inhibitory opsin, we could suppress the soma activity effectively and successfully separate the spine signals from the BAP signals.

Using this method, we conducted large-scale spine imaging (~1,000 spines per neuron) from layer 2/3 pyramidal neurons in mouse primary visual cortex and obtained new findings. First, functional architecture of dendrites can be classified into three synaptic input patterns: dendrites with clusters of spines of similar responses, dendrites with spines of diverse responses, and dendrites with spines where the majority of them show no visual response. Next, to predict the sharp output tuning, we examined several theoretical models based on our experimental data and found that the dendritic cluster model, which only considers the dendrites with cluster of spines of similar responses for the control of output signal, is effective in predicting the sharp output tuning. Our data suggest that only a small fraction of spines (6.9% of the total spines) form clusters of similar inputs on a small subset of dendrites (21.9% of total dendrites), and this functional architecture effectively contributes to the generation of sharply tuned output.

Results

Prevention of BAP by inhibitory opsin

First, to analyze how severely the signals of synaptic inputs are affected by BAP signal along dendrites, we sparsely expressed the genetically encoded calcium indicator GCaMP6s in layer 2/3 pyramidal neurons (see Methods) and imaged the activity of individual spines in response to drifting gratings (Figure S1). GCaMP6s was expressed throughout the dendrites and spines without filling the nucleus of the soma (Figure 1b [DOI](#), Figure S3a). The time course of calcium signal changes in dendrites closely resembled those of soma (Figure S1c, see colored single traces),

suggesting that action potentials propagated back to dendrites. We noticed that spine signals also resembled those of soma and dendrites, suggesting that BAP signals invade individual spines and mask the original spine signals (Figure S1c, colored single traces).

To prevent contamination of the BAP signal in the spine signal, we tried to suppress somatic activity using an inhibitory optogenetic method. To this end, we expressed the bi-stable (step-function) inhibitory opsin SwiChR++ (Berndt et al., 2016 [DOI](#)) in excitatory neurons. To confine the inhibitory effect to the soma, we added a soma-targeting signal to the opsin to localize its expression (Lim et al., 2000 [DOI](#); Figure S2). We expressed GCaMP6s and SwiChR++ with adeno-associated virus (AAV) vectors using the Cre-loxP recombination system and photo inhibited somatic activity of neurons that co-expresses GCaMP6s and mCherry with a laser (Figure 1a, b [DOI](#)). First, we attempted two-photon activation of SwiChR++ with pulsed lasers at wavelengths of 920 nm or 1,040 nm, but neither raster scans nor tornado scans restricted on the soma failed to suppress action potentials (data not shown). We next attempted one-photon activation of SwiChR++ using a visible light laser. Photoinhibition by a raster scan with a visible laser confined to the soma (wavelength: 458 nm, beam diameter: 0.53 μ m, scan size: 20 x 20 pixels (0.5 μ m/pixel), scan speed: 100 ms/frame, duration: 100 s, laser power: 50 μ W after the objective lens) successfully prevented visually evoked somatic activity (Figure 1c [DOI](#), Before-inhibition: mean p(resp)=1.02e-11, mean maximum response=0.562, After-inhibition: mean p(resp)=0.352, mean maximum response=0.013 from n=6 cells respectively, see Methods for the responsiveness criteria). The effect of one-time photoinhibition lasted for approximately an hour, and visually evoked spine activity could be measured without BAP contamination during this time period (Figure S3). By repeating the inhibition of the somatic activity, imaging can be extended to longer time period than previously reported BAP-inhibition methods, patch-clamping the cell body (Jia et al., 2010 [DOI](#); Chen et al., 2011 [DOI](#)) or microinjecting a sodium channel blocker into the soma (Levy et al., 2012 [DOI](#)). Note that, the somatic tuning was measured before photoinhibition of the soma activity, so the measurement of the somatic tuning was not affected by photoinhibition.

The validation of new technique to record spine activity without BAP signals

In order to validate our new technique for recording spine activity, we examined the effect of photoinhibition on the surrounding neurons of the targeted neuron and on the photoinhibited neurons' own spine activity.

First, we investigated the impact of silencing a targeted neuron on the activity of neighboring neurons. Even if the light irradiation is restricted to a single neuron, this alteration has the potential to impact the circuit in which the neuron is embedded, especially its recurrent networks. We expressed a long-lasting inhibitory optogenetic protein (SwiChR++) in transgenic mice (Thy1-GCaMP6s, GP4.3) expressing GCaMP6s in the V1 and compared the response changes around the targeted neuron (250 μ m radius) before and after photoinhibition. For the evaluation, we measured signal changes, preferred orientation and sharpness of orientation tuning (gOSI) as a function of distance from the targeted neuron at 3 different planes (light targeted plane and 30 μ m upper and 30 μ m lower to the light targeted plane). We observed statistically not significant response changes more than 50 μ m away of the targeted neuron, but the response signal was slightly reduced in neurons within 50 μ m radius of the targeted neuron (about 18% decrease on average; 368 orientation-selective neurons from 3 mice) on the same plane as the targeted plane (Figure S4, S5). Despite of the effect on signal changes, there was no significant difference in Δ Ori (the difference of the preferred orientation between before and after inhibition) or gOSI even within 50 μ m radius of the targeted neuron (Figure S6-S9). The same results were obtained on the different planes, upper and lower planes of the targeted planes (Figures S4, S6, S8; upper plane (+30 μ m), 360 orientation-selective neurons from 3 mice; lower plane (-30 μ m), 384 orientation-selective neurons from 3 mice).

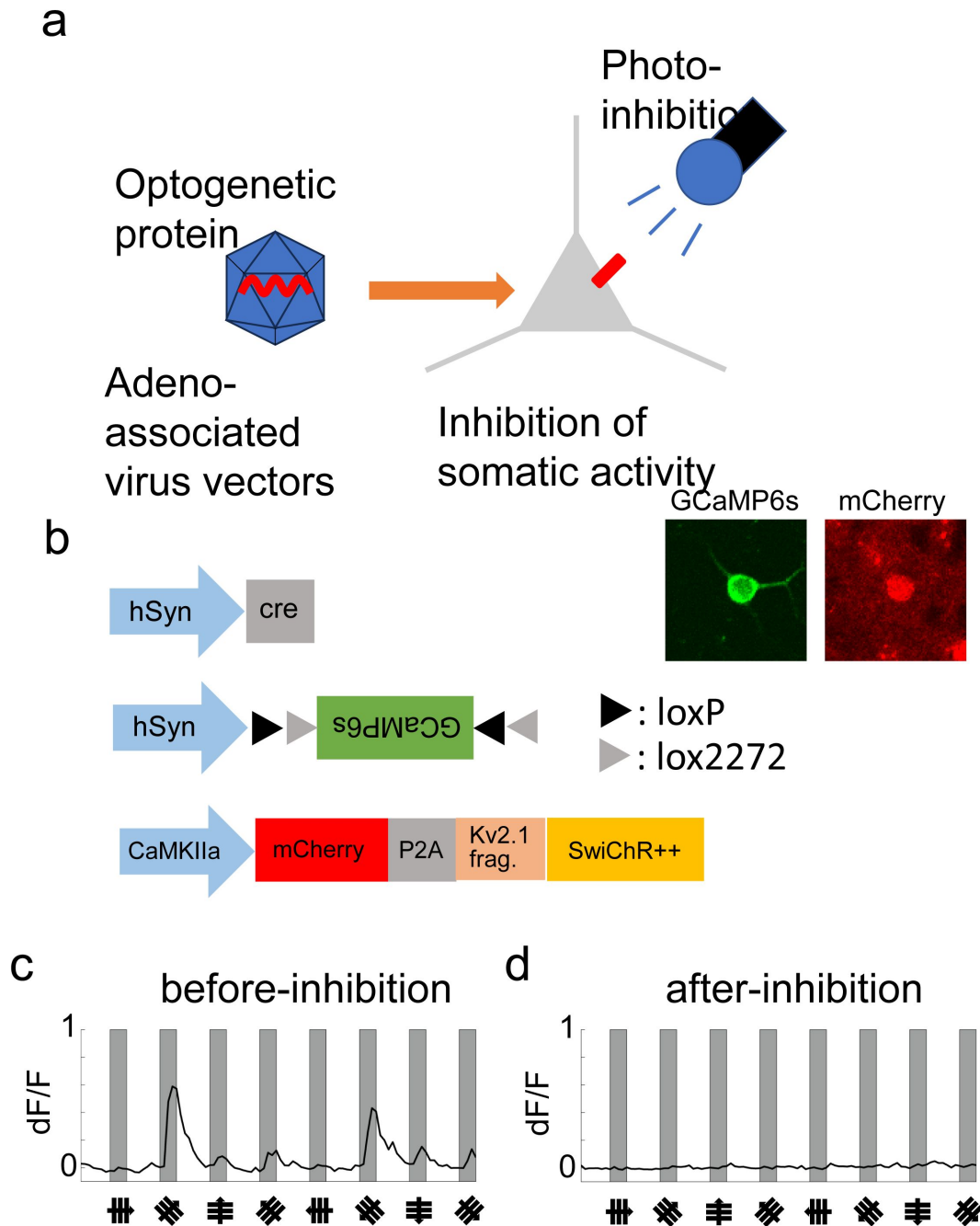


Figure 1.

New method to record visual response of spines without BAP signals.

a. A gene encoding the inhibitory optogenetic protein SwiChR++ is introduced by AAV into layer 2/3 pyramidal neurons in mouse V1. **b.** GCaMP6s is used for the functional spine imaging. Cre-loxP system is used for the sparse expression of GCaMP6s by diluting the Cre-expressing vector. SwiChR++ is used to inhibit the somatic activity. A mixture of three types of AAV is injected to V1. A neuron that expresses both GCaMP6s and mCherry which is the fluorescence marker for the expression of SwiChR++ is shown. **c.** Before photoinhibition, soma shows orientation-selective responses (p value for the response = 2.01×10^{-8} and Max ratio change = 0.6057, see Methods for the definition). **d.** After photoinhibition, the visual response of soma is significantly abolished (p-value for the response = 0.097 and Max ratio change = 0.0129, see Methods for the definition).

Considering the effect of this small reduction (~18%) of the response signal within 50 μm on synaptic inputs, previous studies examining the probability of connections between pyramidal neurons in layer 2/3 of the rat visual cortex showed that while the probability of connections between neurons within 50 μm was high, the absolute number of neurons connected was only about 16% of the total number of connected neurons (10 out of 61) due to the small spatial volume, and about 84% of neuronal connections (51 out of 61) were made with pyramidal neurons more than 50 μm apart (Holmgren et al., 2003). Therefore, it is likely that the slight suppression within 50 μm (~18%) in the present study represents only a small proportion of the total input (~16%) and the slight suppression of these neurons (~18%) is unlikely to have a significant effect on synaptic inputs to the photoinhibited neuron as well.

Next, we examined the effect of soma silencing on the photoinhibited neurons' own spine activities (**Figure 2**, Figure S11; 218 orientation-selective spines from 5 mice). Photoinhibition significantly suppressed action potentials and BAPs (**Figure 1c**). Spines whose preferred orientation is different from the soma are mostly unaffected by BAP. By comparing the response signals of these spines before photoinhibition (without subtraction) and after photoinhibition (BAP-inhibited), the effect of photoinhibition on the photoinhibited cell's own spine can be evaluated. When comparing the spine response signals before and after photoinhibition, photoinhibition did not significantly affect the spine response signal (**Figure 2c**, $p=0.86$ paired sample t-test; Figure S11a, $p=0.09$ one sample t-test). These results indicate that somatic hyperpolarization does not significantly affect the photoinhibited cell's own spine activities.

Then, we compared the two different methods, BAP-inhibition and BAP-subtraction methods. When comparing BAP-inhibited spine signals with BAP-subtracted spine signals, there was no statistically significant difference between BAP-inhibition and BAP-subtraction methods in spine response signals (**Figure 2b-d**, Figure S11a, b), preferred orientation (**Figure 2e, f**), or tuning sharpness (**Figure 2g, h**, Figure S11c, d). The same results were obtained for both types of spines whose preferred orientation is different from (not affected by BAP) (**Figure 2c**, $p=0.92$; e, $p=0.29$ paired sample t-test; Figure S11a, $p=0.08$; b, $p<0.001$ one sample t-test) or the same as (affected by BAP) (**Figure 2d**, $p=0.45$; e, $p=0.50$ paired sample t-test; Figure S11c, $p=0.08$; d, $p<0.001$ one-sample t-test) the soma.

These results indicate that somatic hyperpolarization does not affect the spine signal measurement in the photoinhibited cell, and the similar results were obtained in the spine signal measurements between the BAP-inhibited and BAP-subtracted methods. Previously, it was unknown whether the contaminated signal from BAP is linearly added to the spine signal and whether the contaminated signal can be accurately subtracted by a robust linear regression method (Chen et al., 2013; BAP subtraction method). The present study using the new BAP inhibition method clearly demonstrated the similar results between the two different methods. Our newly developed BAP inhibition method not only realized the measurement of the BAP-free spine signal for the first time, but also demonstrated the validity of the conventional BAP subtraction method, the accuracy of which in estimating the true spine signal was unknown.

Functional input map of layer 2/3 pyramidal neurons in mouse V1

To understand how stimulus selectivity is computed in a single neuron, we reconstructed synaptic input maps from layer 2/3 pyramidal neurons in mouse V1 (**Figure 3**). To image spine activity, we used our new method to prevent somatic activity and recorded visually evoked spine signals under BAP-free experimental conditions. Among the recorded neurons (6 neurons, see **Table 1**), two neurons were orientation selective, one neuron was direction selective, and three neurons were both orientation and direction selective. We imaged the activity of ~1,000 spines per neuron, and 21.6% (average from 6 neurons; range: 16.2-26.9%; **Table 1**) of the spines were significantly responsive (see Methods for the definition) to the drifting grating visual stimulus. Among the visually responsive spines, 85.4% (mean of 6 neurons; range: 82.1-90.0%; **Table 1**) of the spines showed orientation-selective responses, and 57.0% (mean of 6 neurons; range: 48.2-74.3%; **Table**

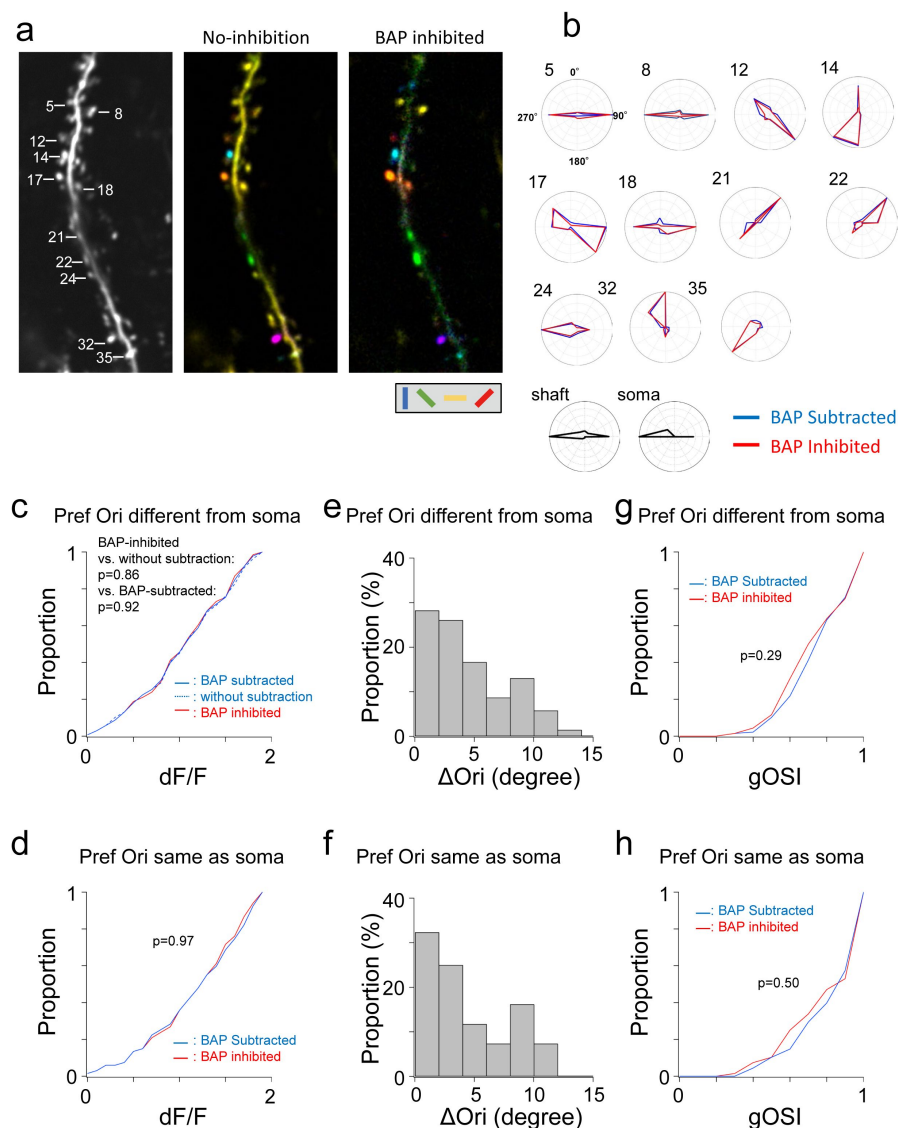


Figure 2.

Comparison between the subtraction and photoinhibition methods for the visually responsive spine activity.

The preferred orientation of the soma of this example neuron is horizontal (coded yellow). **a.** Left: An example image of averaged dendritic branch. Middle: An orientation map of the same dendritic branch without BAP inhibition. Right: An orientation map of the same dendritic branch after BAP inhibition. **b.** Polar plots of visual responses from orientation-selective spines. All the spines show similar orientation preference between BAP-subtracted and BAP-inhibited methods. **c.** Cumulative plots of dF/F of all the orientation-selective spines with preferred orientation different from the soma of without subtraction, BAP-subtracted and BAP-inhibited methods. There is no statistically significant difference among different methods ($p=0.86$, without subtraction vs. BAP-inhibited; $p=0.94$, BAP-subtracted vs. BAP-inhibited; $p=0.92$, without subtraction vs. BAP-subtracted; paired-sample t-test). **d.** Cumulative plots of dF/F of all the orientation-selective spines with preferred orientation same as the soma of BAP-subtracted and BAP-inhibited methods. There is no statistically significant difference between two methods ($p=0.45$ paired-sample t-test). **e, f** Bar plots of difference of preferred orientation (ΔOri) between BAP-subtracted and BAP-inhibited spines with preferred orientation different from the soma (**e**) and same as the soma (**f**). **g.** Cumulative plots of gOSI of all the orientation-selective spines with preferred orientation different from the soma of BAP-subtracted and BAP-inhibited methods. There is no statistically significant difference between two-methods ($p=0.29$ paired-sample t-test). **h.** Cumulative plots of gOSI of all the orientation-selective spines with preferred orientation same as the soma of BAP-subtracted and BAP-inhibited methods. There is no statistically significant difference between two-methods ($p=0.50$ paired-sample t-test).

1 [\[link\]](#) of the spines gave direction-selective responses (see Online Methods for the definition). The distributions of preferred orientation and direction of spines are shown in **Figure 4a** [\[link\]](#). As previously reported ([Chen et al., 2013](#) [\[link\]](#); [Wilson et al., 2016](#) [\[link\]](#); [Iacaruso et al., 2017](#) [\[link\]](#); [Scholl et al., 2021](#) [\[link\]](#)), the proportion of spines with a preferred orientation similar to the soma ($\Delta\text{Ori} \leq 30^\circ$) was higher than the number with other orientations (60.0%, average of 6 cells; range: 44.7–77.0%; **Figure 4b, c** [\[link\]](#)).

Predicting somatic tuning from the number of responsive spines

To investigate whether the soma response could be estimated by linear summation of the spine responses, we first averaged the time courses (**Figure 5a** [\[link\]](#)) or tuning curves (**Figure 5b** [\[link\]](#)) of the calcium signals from all recorded spines and compared them with the soma response. By averaging the signals from all spines, we estimated the orientation tuning curve of the spine inputs, which was able to predict the preferred orientation of the soma but was broader than that of the soma (**Figure 5** [\[link\]](#)). To assess the tuning sharpness and similarity between the signals of the soma and the averaged spines, we calculated the orientation selectivity index (OSI) and the correlation index (CI) (see Online Methods for the formula, $\text{CI} = 1$ means a perfect match between the two tuning curves). The OSI showed a much broader tuning in the averaged spines signal ($\text{OSI} = 0.63$) than in the soma signal ($\text{OSI} = 1$). The CI ($\text{CI} = 0.69$) indicated that the tuning curves between the soma and the averaged spines were not very similar. These results suggest that the simple summation of the spine responses is not a good predictor of somatic tuning.

The time course of signal changes in spines includes weak, subthreshold responses, and simple summations of spine responses may have broadened the tuning curve. To exclude the weak and subthreshold spine responses, instead of averaging the responses of all spines, we summed the number of all spines that significantly responded to the visual stimulation for each stimulus direction (Figures S11c, S12a). The polar plot showed that the tuning curve of the summed spines only slightly approached the tuning curve of the soma compared to the simple summation of the spine responses. When we compare the ‘Figure S12b’ (summation of dF/F) and ‘Figure S12c’ (summation of number of responded spines), CI slightly increased from 0.69 to 0.78, and the preferred direction became the same as the soma (the preferred direction of ‘Figure S12b’ is opposite to the soma), however the overall tuning curve of both are still dissimilar to that of soma. The increase of the response threshold did not further improve the CI (Figure S13a, d).

Since this summation is only a digital summation (0 or 1), we investigated incorporating a weighting for individual spines to improve the estimation of the somatic tuning. First, spine activity was weighted by the change in calcium signal (Figures S11d, S12b). The prediction of somatic tuning was not significantly improved compared to the result of unweighted summations ($\text{CI} = 0.78$ (unweighted) to 0.80 (weighted by calcium signal change)). Improvement of CI was not observed even if we increased the response threshold of summation (Figure S13b, d).

The effect of spine activity on the soma may depend on the distance of the spine from the soma, and the closer the spine is to the soma, the greater the effect ([Spruston, Jaffe & Johnston, 1994](#) [\[link\]](#)). Therefore, we next considered the distance between the soma and the spines as a weighting factor. The weighting coefficient was defined as ‘ $\exp(-\text{distance}/\text{length constant})$ ’, which is derived from the cable theory of dendrites ([Rall, 1959](#) [\[link\]](#)). Based on the literature for layer 2/3 pyramidal neurons ([Larkum et al., 2007](#) [\[link\]](#)), the length constant was set to 200 μm (Figure S12e). The predicted tuning curve was no better than without weighting (Figure S12c; $\text{CI} = 0.78$, both unweighted and weighted by distance from the soma). We also calculated the tuning curves while changing the length constant from 100 μm to 300 μm , but there was no difference in the tuning curves obtained (Figure S14a, b).

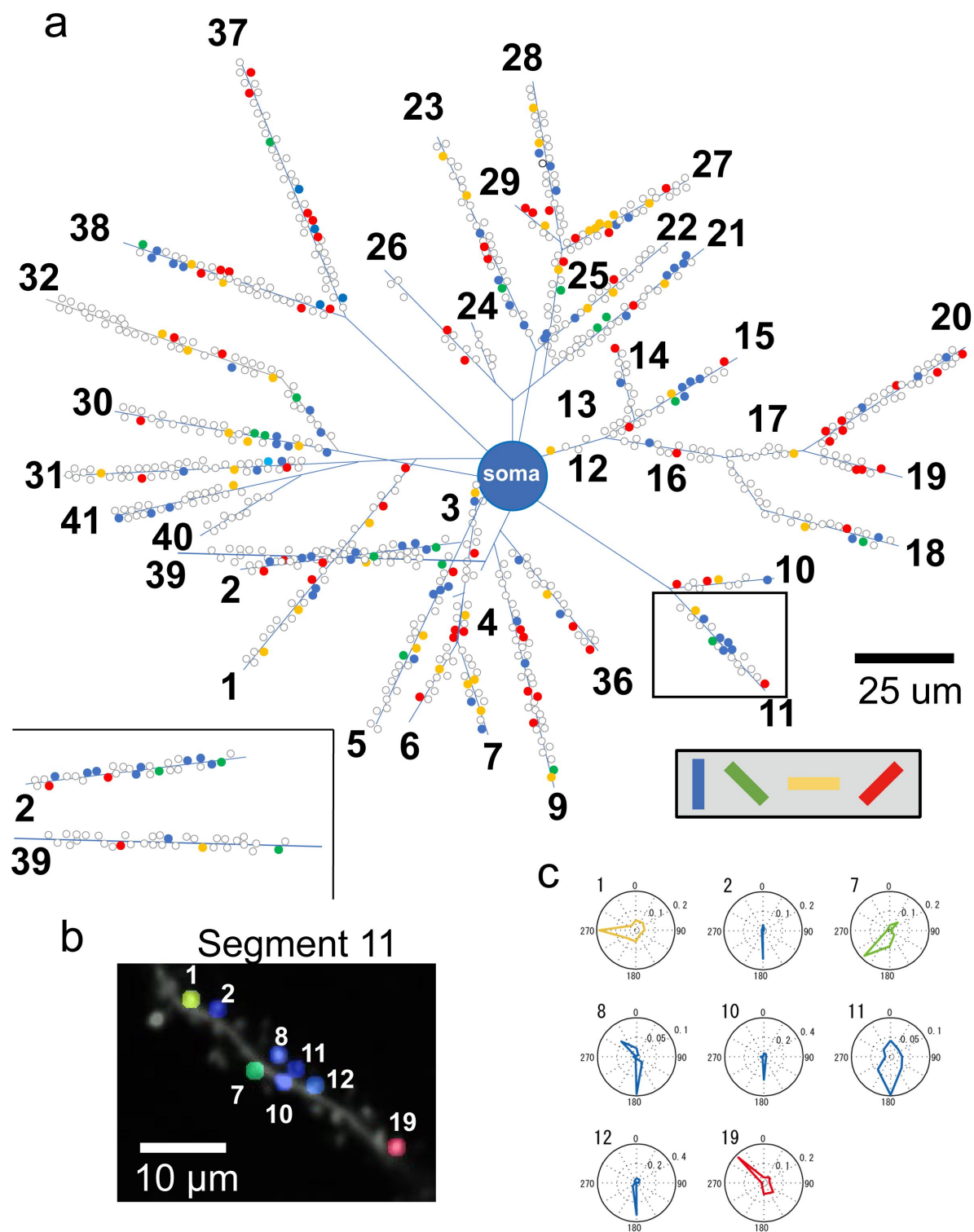


Figure 3.

Functional spine map of a layer 2/3 pyramidal neuron.

a. A functional spine map from ~1,000 spines recorded from the basal dendrites of neuron 1. Orientation-selective spines are illustrated by different colors, and uncolored spines are either visually unresponsive or visually responsive but nonselective. **b.** A spine orientation map from branch 11 of neuron 1 (see [Table 1](#)). Among nineteen spines recorded from this branch, eight spines selectively responded to orientation stimulation. **c.** Polar plots of orientation-selective spines in branch 11.

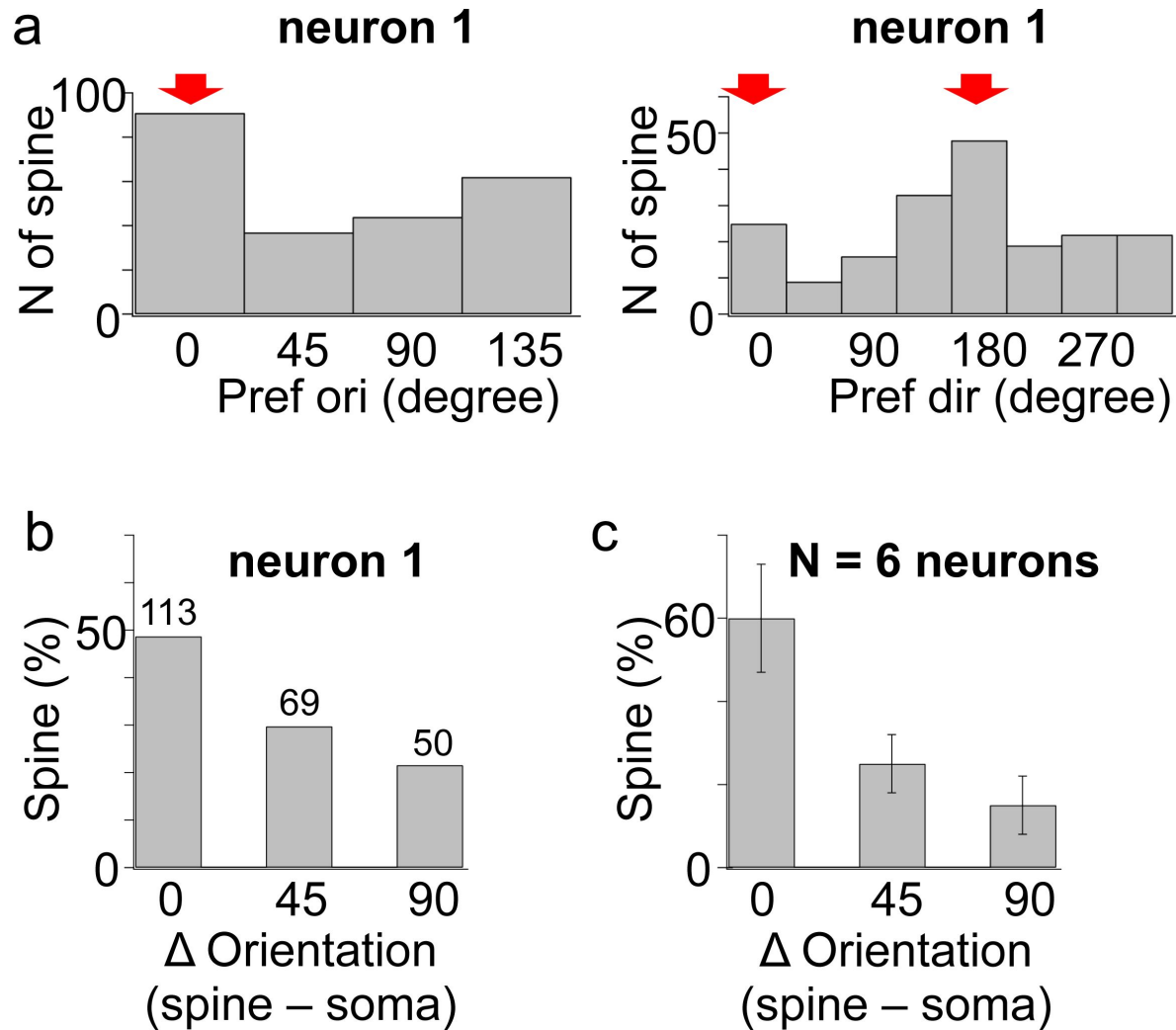


Figure 4.

The preferred orientation of the soma can be predicted from the number of orientation-selective spines.

a. Distributions of preferred orientations (left) and directions (right) of spines in neuron 1. The peaks of the distribution match the preferred orientation and direction of the soma (red arrow). **b.** The distribution of the difference in preferred orientation between each spine and soma (Δ Orientation) of neuron 1. About half of spines are tuned similarly to the soma. **c.** Averaged data from 6 neurons shows the same trends as neuron 1 data.

Neu ron #	Soma OSI	Soma DSI	Recorded spine N	N of visually responded spine	N of recorded segment	N of orientation- selective spine	N of direction- selective spine
1	1	0.45	996	261 (26.2%)	41	234 (90.0%)	194 (74.3%)
2	1	0.51	920	217 (23.6%)	32	188 (86.6%)	119 (54.8%)
3	1	0.01	723	117 (16.2%)	24	96 (82.1%)	62 (53.0%)
4	1	0.25	993	198 (19.9%)	41	165 (83.3%)	115 (58.1%)
5	0.32	0.65	1119	301 (26.9%)	40	251 (83.4%)	162 (53.8%)
6	1	0.65	982	166 (16.9%)	30	144 (86.7%)	80 (48.2%)

Table 1

Summary of recorded spines

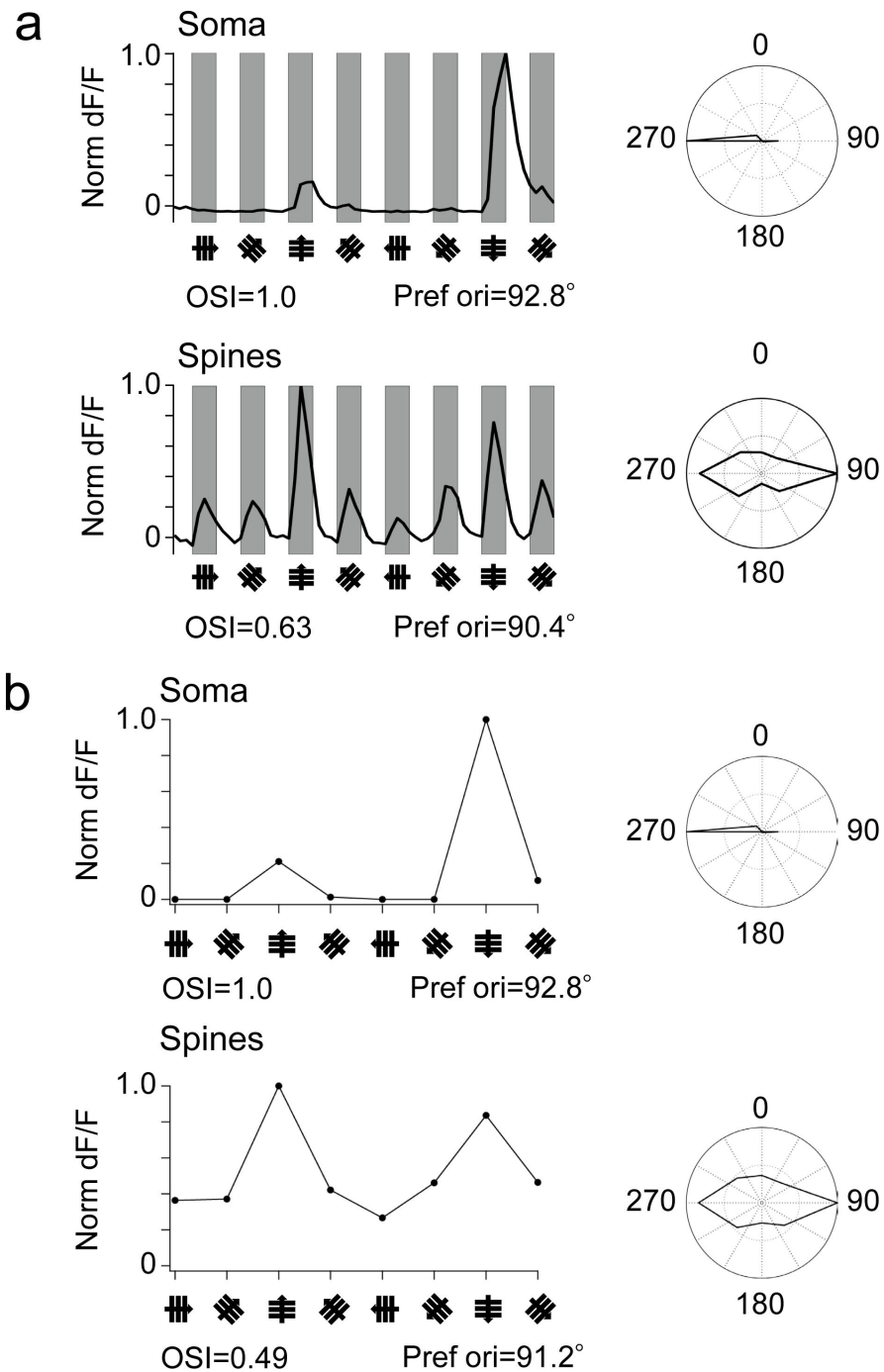


Figure 5.

Summation of calcium time courses of spines can predict the preferred orientation of the soma, but with the broad tuning curves.

a. The time course of the soma (upper panel; average of 10 trials) and the average of the spine signals (lower panel; average of 10 trials from 982 spines). The OSI and preferred orientation are shown for both datasets. Polar plots suggest the broader tuning of the average of the spine signals than the tuning of the soma. **b.** The tuning curve of the soma (upper panel; average of 10 trials) and the average of the spine tuning curves (lower panel; average of 10 trials from 982 spines). Polar plots suggest the broader tuning of the average of the spine tuning curves than the tuning curve of the soma. Although the OSI from the average of the spine signal and tuning curve is lower than that of the soma, the preferred orientation can be effectively predicted in both cases.

We also tested the possibility that spines located within a limited distance from the soma could effectively contribute to the somatic tuning. We selected the spines as a function of distance from the soma (range 100-300 μm ; step size 50 μm) and calculated the tuning curve using the same method (Figure S14c, e). When only spines within 150 μm from the soma were selected, the CI did not improve very much (CI = 0.82 within 150 μm) and the results were still far from reproducing the tuning of the soma (Figure S14e). In addition, when only spines above 150 μm from the soma were selected, the CI was smaller than the value of within 150 μm (CI=0.67 above 150 μm). Spines with a similar preferred orientation to the soma showed no apparent distributional bias with distance from the soma (Figure S14d). Taken together, the tuning curve estimated by a simple summation of the number of responding spines was not improved by considering the weighting factors of either the spine signal change or the electrotonic length constant.

Two forms of functional clustering of similarly tuned spines on the dendritic branch

It has been suggested that, given the impedance matching at the branch point, each dendritic branch may be an electrical compartment for the local processing of input signals and that branch-specific computation may occur (Branco & Hausser, 2010). We considered dendritic branches as the integrating unit of synaptic inputs and focused our analysis on dendritic branches (see Discussion). Recent studies have suggested that nonlinear local summation of clustered synaptic inputs on dendrites may influence somatic spiking (Jia et al., 2010 [DOI](#); Wilson et al., 2016 [DOI](#); Smith et al., 2013 [DOI](#); Major et al., 2013 [DOI](#); Palmer et al., 2014 [DOI](#); Bicknell & Hausser, 2021).

Our reconstructed functional input maps (e.g., **Figure 3a, c** [DOI](#)) indicated the clustering of similarly tuned spines in some branches. Therefore, we quantitatively analyzed the spatial location and orientation tuning of spines on each dendritic branch. First, we calculated the distance and the difference in preferred orientation between the spines in each branch (**Figure 6** [DOI](#)). We found that the ΔOri of nearby spines (< 3 μm apart) in the same dendritic branch was smaller in the actual data than in the shuffled data (shuffled all spines across cell, $p=0.023$ Mann-Whitney U-test with the Bonferroni correction; shuffled all spines within a segment, $p=1$ Mann-Whitney U-test with the Bonferroni correction). The results indicate the presence of functional clustering of spines at the spatial scale of less than 3 μm distance between spines (spine-pair level). Next, we counted the number of spines that responded to each orientation stimulus on individual dendritic branches (Figure S15). So far, we found two forms of functional clustering of similarly tuned spines on the dendritic branch, one is clustering at the scale of neighbor spine-pairs level and the other is the clustering at the scale of the dendritic branch level.

We observed clusters of spines with similar responses on a small subset of dendritic branches, but most dendritic branches have either diversely responsive or visually unresponsive spines (Figure S15; see also **Figure 3** [DOI](#)). The proportion of visually responsive spines on each dendritic branch is highly variable, ranging from 0% to 100% in our recorded data (average 20.7% among 208 dendritic branches from 6 neurons). Our functional synaptic input maps (e.g. **Figure 3** [DOI](#)) suggest that the dendritic branches can be classified into three patterns of synaptic input architecture.: branches with clusters of spines of similar responses, branches with spines of diverse responses, and branches with spines where the majority of them show no visual response.

Relationship between two forms of functional clustering

We conceptualized two forms of functional clustering, one at the level of spine pairs (**Figure 6** [DOI](#); the distance between two spines is less than 3 μm) and the other at the level of dendritic branches (Figure S15). We analyzed the relationship between the branch-level clustering and the spine-pair-level clustering. To investigate the relationship between branch-level clustering and spine-pair-level clustering, we evaluated the contribution of branch-level clustering to spine-pair-level clustering. We calculated the proportion of similarly tuned spine-pairs to all spine-pairs at different distances of paired spines (30 μm length in 3 μm steps) on each branch and compared it

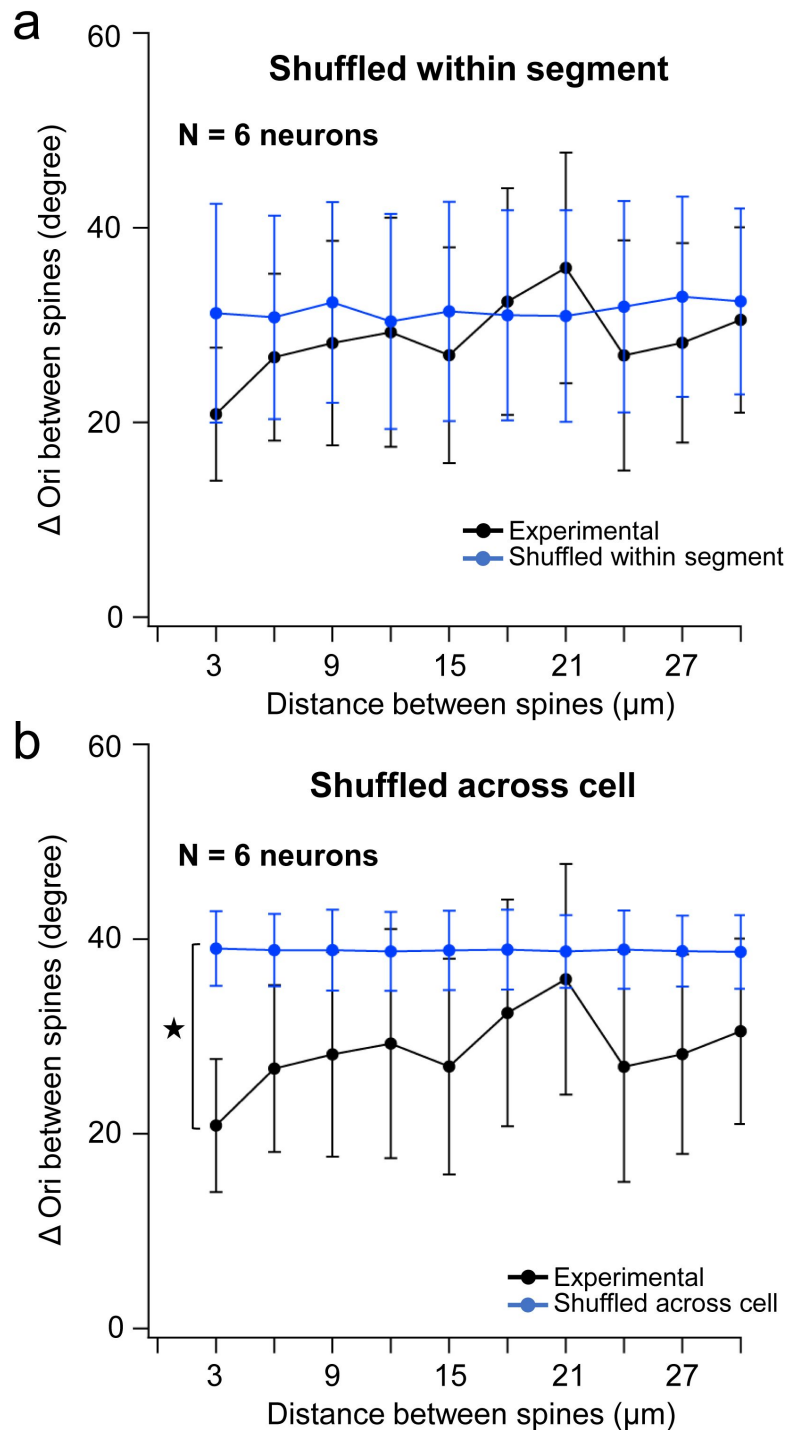


Figure 6.

Functional clustering at the spine-pair level

The difference in preferred orientation (Δ Orientation) are plotted as a function of the distance between spines on each dendritic branch and the actual data (**a**, **b**, black line) are compared with the shuffled data (**a**, **b**, blue line). In the shuffled data, all the pines were shuffled, while maintaining the original spine locations and the same proportion of orientation-tuned spines. Shuffling was done either across cell (**a**, blue line) or within each dendritic branch (**b**, blue line). Δ Orientation between neighboring spines ($< 3\mu\text{m}$) of actual data is significantly smaller than that of shuffled data across a cell (**a**, $p = 0.023$ Mann-Whitney U-test with the Bonferroni correction) and smaller but not significantly different than that shuffled within a branch (**b**, $p=0.83$ Mann-Whitney U-test with the Bonferroni correction).

between the experimental data and the randomized data (Figure S16). To compute the randomized data, 1,000 new functional spine input maps were generated for each neuron. Randomization was performed either within a branch (Figure S16a blue line) or across a cell (Figure S16a red line). The randomized maps contained the same proportion of visually responding spines, and the response properties of all spines were randomly shuffled in each analysis, preserving the original spine positions.

The proportion in the bin smaller than 3 μm represents the proportion of spine-pair-level clustering. We found that only a small proportion of spine pairs were similarly tuned (see Figure S16b, height of black bar: $\sim 4.7\%$, ~ 44 spine pairs on average from 6 neurons). Spine-pair-level clustering can be explained by the spine input pattern in the branch, branch-level clustering, and the overall proportion of similarly tuned spine inputs. Randomizing all spines within a branch significantly reduced the proportion of similarly tuned spine pairs (see Figure S16b, difference in height between black and blue bars: 1.8% , $p=0.026$ Mann-Whitney U-test with the Bonferroni correction), and this reduction may correspond to the contribution of specific spine positions within the clustered branch (Figure S16a, b). Randomizing all spines across cell further significantly reduced the proportion of similarly tuned spine pairs (see Figure S16b, difference in height between blue and red bars: 2.5% , $p<0.001$ Mann-Whitney U-test with the Bonferroni correction), and this reduction may correspond to the contribution of branch-level clustering (Figure S16a, b). Thus, it can be estimated that branch-level clustering may contribute about half to the spine-pair-level clustering.

Predicting somatic tuning from clustering of spines on dendrites and thresholding at soma

We tested a cluster model in which clustering of similarly activated spines can sharpen the broad tuning predicted by a simple summation of activated spines. In this model, we assumed that only when the number of responsive spines in a dendritic branch exceeds a clustering threshold do these spines contribute to the depolarization of the soma (Figure 7a). As the cluster threshold increased, the proportion of the spines responding to the same preferred orientation as the soma (Figure 7a, red line) among the spines responding to any orientation (Figure 7a, black line) increased (Figure 7a, blue line). Thus, as the threshold increased, the predicted time course became tighter (Figure 7b) and the predicted soma selectivity became sharper (Figure 7c).

On the other hand, it has been suggested that whether the soma evokes action potentials or not is controlled by the nonlinear effect of the spike threshold in the soma (Priebe & Ferster, 2008). Therefore, we investigated another model to predict the soma tuning. We considered a threshold at the soma (soma threshold) for triggering spikes and investigated whether the prediction of the somatic tuning could be improved when the soma threshold was varied (a threshold model, Figure 7d-f). In this model, the soma threshold is the number of responding spines that trigger a spike in the soma. As the soma threshold increased, the proportion of the spines responding to the preferred orientation of the soma among the spines responding to any orientation increased (Figure 7d). Thus, as the threshold increased, the predicted time course became tighter (Figure 7e) and the predicted soma selectivity became sharper (Figure 7f). These results indicate that both the cluster and soma threshold models are effective models for predicting the soma tuning.

Previous model study suggested the two-layer input-output function of the pyramidal neuron, that is, synaptic input summation at the dendrite occurs in the first layer and thresholded final response at the soma is produced in the second layer (Poirazi et al., 2003). This model has been experimentally tested and revealed that combination of dendritic nonlinearity and spike thresholding contribute to the sharpness of orientation tuning in the ferret visual cortex (Wilson et al., 2016). Therefore, we tested this model using our experimental data if we see further improvement of tuning also in the mouse (Figure 8). The heat map of CI showed that the highest

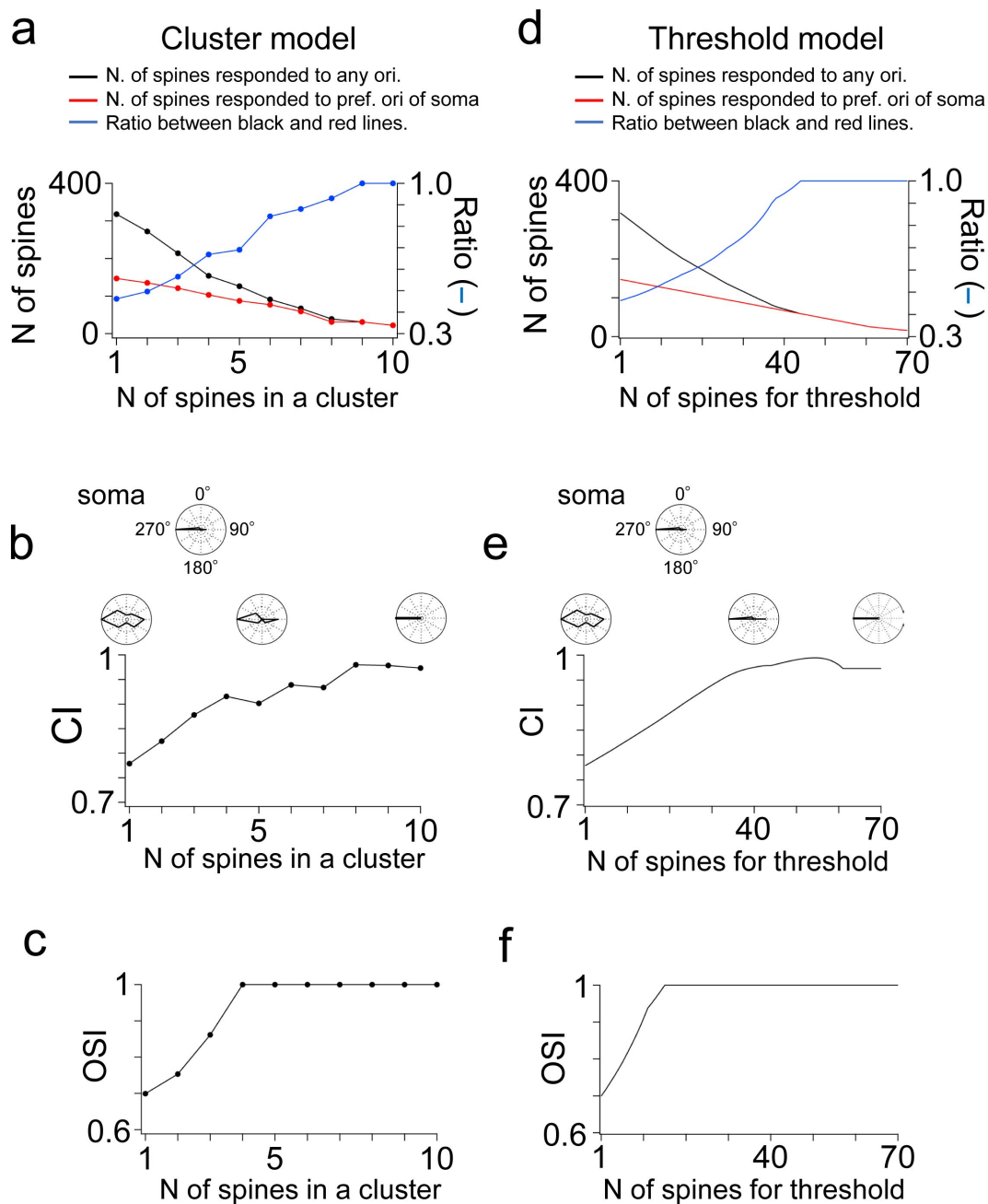


Figure 7.

The prediction of somatic tuning was improved by the cluster model on the dendrites and the threshold model at the soma

a. The number of responsive spines is plotted as a function of the number of spines in a cluster. The black line shows number of spines responding to any orientation, and the red line shows number of spines responsive to the preferred orientation of the soma. The blue line represents the ratio between the red and black lines. **b.** The CI between the actual and predicted tuning curves of the soma plotted as a function of the number of spines in a cluster. Polar plots of the actual and predicted tuning curves of the soma are shown. **c.** The OSI plotted as a function of the number of spines in a cluster. **d.** The number of responsive spines is plotted as a function of the number of spines for the threshold. Black line shows number of spines responding to any orientation, and red line shows number of spines responsive to the preferred orientation of the soma. The blue line represents the ratio between the red and black lines. **e.** The CI between the actual and predicted tuning curves of the soma plotted as a function of the number of spines for the threshold. Polar plots of the predicted tuning curve of the soma are shown. **f.** The OSI plotted as a function of the number of spines used as a threshold.

CI (CI = 1.00) was obtained at the combination of cluster size 8 and soma threshold 7 (**Figure 8a** [↗](#), left), and this combination accurately predicted the soma tuning (**Figure 8a** [↗](#), right). Another example of a neuron showed a similar improvement in the estimation of the somatic tuning (**Figure 8b** [↗](#), CI = 0.99, cluster size 6 and soma threshold 8). A similar trend was observed in the population average of the individual heat maps of the six neurons (**Figure 8c** [↗](#)). The parameters that best predicted the soma tuning varied from about 4 to 6 spines for the cluster size and from about 5 to 20 spines for the soma threshold (**Figure 8c** [↗](#), CI = 0.95, cluster size 6 and soma threshold 6). These results suggest that both local integration in dendrites and global integration at the soma sharpen the broad tuning and determine the fine tuning of the soma (Figure S17).

Small number of clustered branches and spines are sufficient to reproduce the tuned output signal

Our synaptic input integration model is able to predict soma output tuning even though it used only dendritic branches containing clustered synaptic inputs (6-8 spines/branch) that responded to the same preferred soma orientation, ignoring other dendrites (**Figure 9f** [↗](#)). This means that the branches with small number of spines can be considered as functional clusters, suggesting that they are critical branches for soma output tuning. We analyzed the number and the spatial distribution of these clustered dendrite within individual neuron (**Figure 9a-e** [↗](#)). The results showed that the number of branches averaged 7 per neuron (**Figure 9b** [↗](#); 21.9% of all branches: 12.2%~31.2% from 6 neurons) and the number of clustered spines averaged 63 per neuron (**Figure 9c** [↗](#); 6.9% of all spines: 4.7%~8.5% from 6 neurons). These dendritic branches are distributed throughout the dendrites without bias (**Figure 9a** [↗](#)). These dendrites distributed most abundant 50-100 μm from the soma (**Figure 9d** [↗](#), 30 of 44 branches in 6 neurons, approximately 68% of the total) and in the second and third branches (**Figure 9e** [↗](#), 36 of 44 branches in 6 neurons, approximately 82% of the total).

Discussion

In this study, we first established a new method to prevent BAP signal along dendrites, which has been the major obstacle to accurately recording of signal changes in individual spines. Using this method, we conducted large-scale spine imaging (~1,000 spines per neuron) from layer 2/3 pyramidal neurons in mouse primary visual cortex and obtained new findings. First, functional architecture of dendrites can be classified into three synaptic input patterns: dendrites with clusters of spines of similar responses, dendrites with spines of diverse responses, and dendrites with spines where the majority of them show no visual response. Next, to predict the sharp output tuning, we examined several theoretical models based on our experimental data and found that the dendritic cluster model, which only considers the dendrites with cluster of spines of similar responses for the control of output signal, is effective in predicting the sharp output tuning. Our data suggest that only a small fraction of spines (6.9% of the total spines) form clusters of similar inputs on a small subset of dendrites (21.9% of total dendrites), and this functional architecture effectively contributes to the generation of sharply tuned output.

New method to record spine activity by preventing BAP

Imaging of spine activity under the hyperpolarization of somatic membrane potential to prevent BAP has previously been performed by patch-clamping the cell body (Jia et al., 2010 [↗](#); Chen et al., 2011 [↗](#)) or microinjecting a sodium channel blocker into the soma (Levy et al., 2012 [↗](#)). In contrast, in this study, we hyperpolarized the somatic membrane potential by using a long-lasting bistable inhibitory opsin (Berndt et al., 2016 [↗](#)) with a soma-targeting signal. Our optogenetic method is technically easier and can maintain the membrane potential longer than electrophysiological or chemical methods to perform large-scale dendritic mapping. On the other

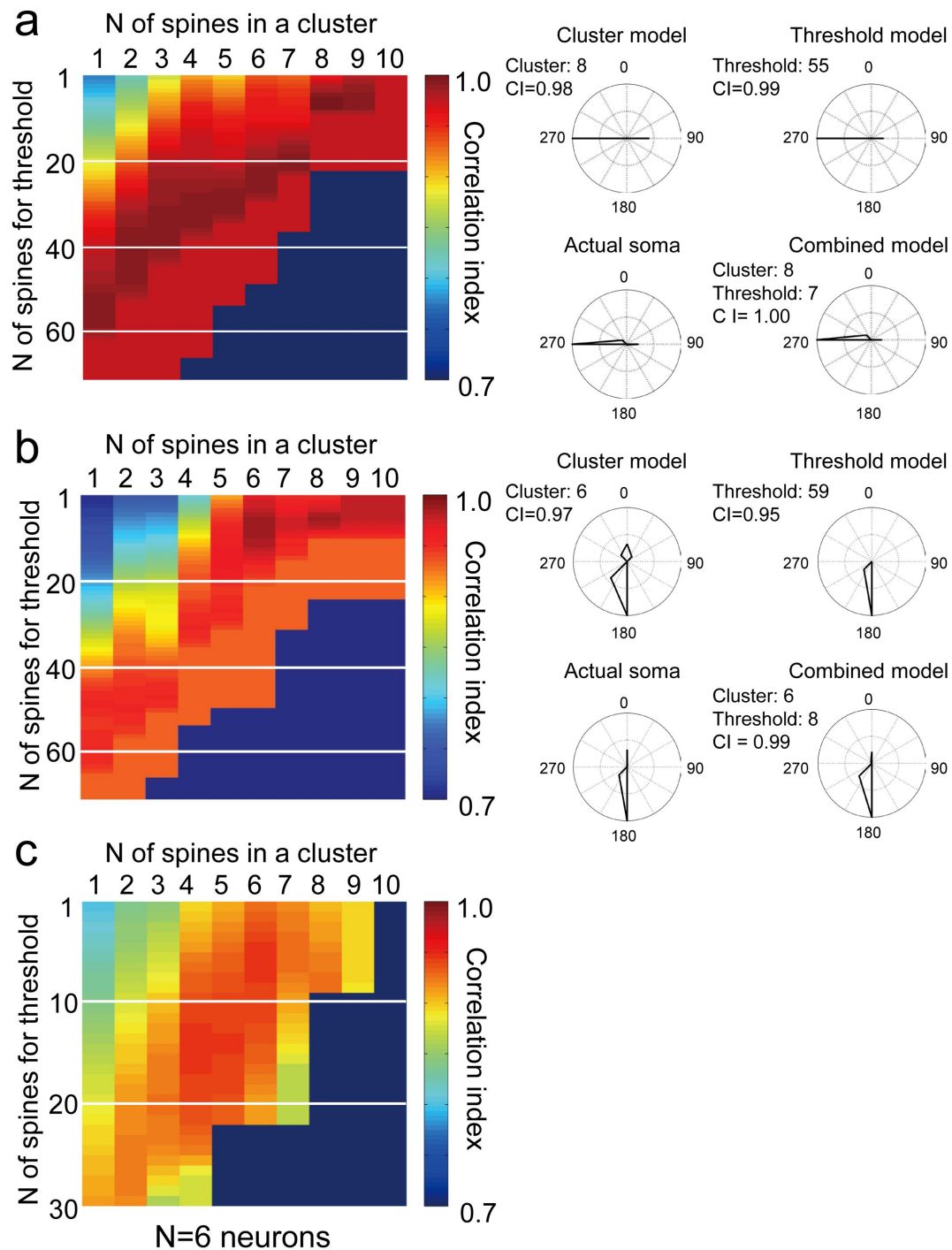


Figure 8.

The combined model further improves soma tuning.

a. A combined model is applied to neuron 5 (see [Table 1](#)). CIs are calculated at various combinations of values for the number of spines (1-10 spines) in a cluster and the number of spines (0-70 spines) used as the threshold, and CI values are shown as a heat map. **b.** Another example of a combined model in neuron 2. **c.** Population averaged data from six neurons. Heat maps are calculated for each neuron and averaged.

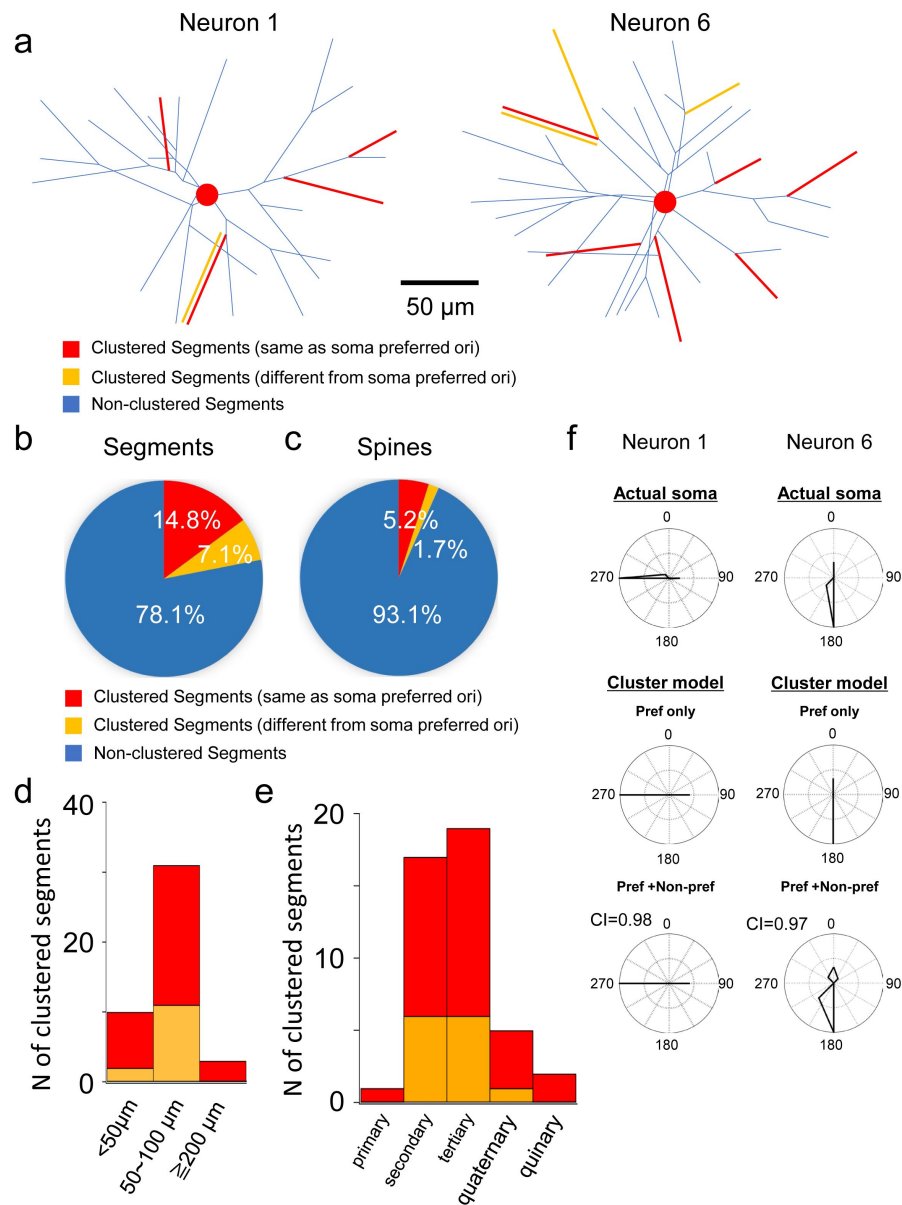


Figure 9.

Analysis of dendritic branches with cluster of spines responding to the similar orientations.

a. Two examples of neuron showing the segments with cluster of spines responding to the same orientation as the soma preferred orientation (red) and different orientation from the soma preferred orientation (yellow). Clustered segments are distributed spatially uniform manner on the dendritic field in both neurons. **b.** Proportion of clustered segments (red and yellow) and non-clustered segments (blue). Among clustered segments (21.9%), the segments of spines responding to the same orientation as the soma preferred orientation (red) is twice as much as the segments of spines responding to the different orientation to the soma (yellow). **c.** Proportion of spines on the clustered (red and yellow) and non-clustered (blue) dendritic segments. Among spines on the clustered segments, the spines responding to the same orientation as the soma preferred orientation (red) is three times as much as the spines responding to the different orientation to the soma preferred orientation (yellow). **d, e.** Distribution profiles of clustered segments on the dendritic field are shown. Number of clustered segments is plotted as a function of the distance from the soma (**d**) and the branch order (**e**). **f.** Prediction of soma tuning using spine responses on the clustered dendrites. Two examples from the same neurons shown in (**a**). upper plot: Actual soma tuning. middle plot: Predicted tuning using spine responses on the clustered segments with the same preferred orientation as the soma. lower plot: Predicted tuning using spine responses on the clustered segments with both the same and the different preferred orientation to the soma. Better prediction was obtained when using the both clustered segments.

hand, there is concern that a continuous increase in chloride conductance due to activation of soma SwiChR++ channels may affect synaptic inputs. Increased chloride ions in the soma may to some extent spread to dendrites. Normally, intracellular chloride levels are regulated by the neuron-specific potassium chloride transporter (KCC2) expressed in both the soma and dendrites (Baldi et al. 2010). However, it is unclear how effectively dendritic KCC2 prevent the spread of chloride ions from the soma to the dendrites in our experimental condition. The lack of difference in the magnitude of synaptic responses before and after photoinhibition at non-preferred orientations (**Figure 2C**), which are thought to be largely unaffected by BAP, suggests that the increased chloride ion permeability of the soma has not significant effect on the dendrites.

Large-scale spine imaging

To understand the tuning mechanisms from synaptic input to soma output, it is necessary to record spine activity from individual neurons as much as possible. It has been previously reported that a single layer 2/3 pyramidal neuron in the cortex possess ~7,000 spines and among them ~4,000 spines are located on basal dendrites (Lascone et al., 2020). In this study, we performed large-scale functional imaging of spines on the basal dendrites. We recorded ~1,000 spine activities from each neuron in response to visual stimulation. We found that on average only 21.6% of the spines are visually responsive to the drifting grating stimulation. It is unexpectedly small that most of the synaptic inputs to layer 2/3 pyramidal neurons in V1 are visually unresponsive. The proportion of visually unresponsive spines was not related to the time course of the recording. For example, in Figure S15, the higher the dendrite number, the later they were recorded from the start, but there is no relationship between the dendrite number and the presence or absence of a visual response. Our results showed that the proportion of visually responsive spines on each dendritic branch is highly variable, ranging from 0% to 100% in our recorded data (average 20.7% among 208 dendritic branches from 6 neurons). This suggests that the synaptic input organization of each dendritic branch is functionally heterogeneous. A small subset of dendritic branches has clusters of spines with similar orientation tuning, while others have spines with heterogeneous orientation tuning or no visual response. The origin and synaptic organization of visually unresponsive inputs to a single neuron is not known well. One of the possible origins could be visually unresponsive surrounding neurons within V1. Previous studies by ours and others have shown that only about half of the excitatory neurons in V1 are visually responsive to the drifting grating stimulus at a given spatial and temporal frequency (about 50–60% in layer 2/3 of V1, Kerlin et al., 2010; Marshel et al., 2011; Kondo et al., 2016). Recurrent connections are mostly formed on the basal dendrites (Spruston, 2008), from which we recorded spine signals in this study. Another possible source of visually unresponsive inputs may be projections from outside V1. Recent retrograde labeling studies have shown that CTB (retrograde labeling tracer) injections into V1 layer 2/3 revealed inputs from many nonvisual brain areas (Morimoto et al., 2021). In summary, a single layer 2/3 pyramidal neuron in V1 may receive mixed synaptic inputs from both visually responsive and visually unresponsive neurons inside and outside of V1, and the dendritic branches may be organized by the spines that receive visually responsive and visually unresponsive synaptic inputs according to a rule that remains to be clarified.

Functional clustering of synaptic inputs on subsets of dendritic branches

We found that the spines evoked by the preferred soma orientation were dispersed across multiple dendritic branches, but clustered on some branches of layer 2/3 pyramidal neurons in mouse V1. Our simulated model suggested that as the number of spines used to define a cluster increased, the predictive accuracy of somatic tuning increased (**Figure 7b**). Large-scale spine imaging allowed us to estimate the number and the spatial distribution of clustered dendritic branches and the number of spines in the cluster. We found that only a small fraction of dendritic branches (~22% of the total number of dendrites) that are spatially distributed can reproduce somatic tuning without considering other dendritic branches. Accordingly, the number of spines

clustered on dendritic branches is also small ($\sim 7\%$ of the total number of synaptic inputs). This type of functional organization may be conserved across species with or without functional columns and different visual functions, such as functional clustering for spatial location in mice (Iacaruso et al., 2017 [DOI](#)), clustering of callosal and non-callosal inputs in mice (Lee et al., 2019 [DOI](#)), and functional clustering of orientation inputs in ferrets (Wilson et al., 2016 [DOI](#)). Functional clustering of synaptic inputs may be effective for strong activation while maintaining robustness against information loss along dendrites (Stuart and Spruston, 2015 [DOI](#)).

Weighted and unweighted summation of activated spines failed to estimate the sharp tuning of the soma

Previous studies (Chen et al., 2013 [DOI](#); Wilson et al., 2016 [DOI](#)) and our study showed that the preferred orientation of the soma can be predicted by the summed number of activated spines or the averaged time course of spine responses, but showed broader orientation tuning. Even when we considered the magnitude of the spine signal change or the distance between the soma and the spine, the estimation did not improve. Regarding the magnitude of spine signal change, calcium signaling is not the direct measure of synaptic transmission via AMPA receptors, but rather via NMDA receptors (Sobczyk et al., 2016 [DOI](#)). Recent work (Scholl et al., 2021 [DOI](#)) suggests that the magnitude of calcium signal change may not be a good predictor of synaptic strength. Regarding the distance between the soma and activated spines, it has been previously shown the distance independence of synaptic inputs on the somatic synaptic integration (Magee, 2000 [DOI](#)). Thus, our result is not surprising given the long length constant ($\sim 300 \mu\text{m}$) of cortical layer 2/3 pyramidal neurons (Larkum et al., 2007 [DOI](#)) and the distance-independent distribution of activated spines with different orientation selectivity (Figure S14d).

Cluster model at the dendrites to predict soma tuning

The spatial arrangement of functional synaptic inputs on the dendrite should provide useful information to reveal the computation performed by dendritic integration. Many theoretical models have been developed to understand the electrical behavior of dendrites (Poirazi & Papoutsi, 2020 [DOI](#)). In the compartmental model, dendrites are divided into multiple compartments, and each compartment is considered as a computational unit (Rall, 1964; Poirazi et al., 2003 [DOI](#)). Dendrites can be divided into compartments in various ways. Among them, considering the impedance matching at the branch point, each dendritic branch can be an electrical compartment for the local processing of input signals, and branch-specific computation could occur (Branco & Hausser, 2010). The electrically isopotential dendritic length at which synaptic inputs can be effectively integrated, is reported to be $\sim 40 \mu\text{m}$ in neocortical pyramidal neurons (Mel, 1993 [DOI](#)). Because the average length of the dendritic branches in our recorded neurons was mostly around $20\sim 30 \mu\text{m}$ (see Methods), we considered the dendritic branches as the integrating unit of synaptic inputs and focused our analysis on the dendritic branches. We observed clusters of similarly tuned synaptic inputs on the dendritic branches of layer 2/3 pyramidal neurons. This feature is particularly efficient for the somatic spiking because unlike spatially distributed coactivated spines, clustered coactivated spines can evoke the dendritic spikes and influence the somatic activity more effectively (Stuart & Spruston, 2015 [DOI](#); Ujfalussy & Makara, 2020 [DOI](#)). Dendritic spikes triggered by coactive synaptic inputs have been observed as dendritic hotspots in the previous studies (Jia et al., 2010 [DOI](#); Wilson et al., 2016 [DOI](#)). In our present study, the cluster model improved the estimation of the soma orientation tuning, suggesting the importance of the clustering of synaptic inputs with similar orientation tuning. The dendritic hotspots were not observed in our recording, probably because the strong hyperpolarization of the soma with the optogenetic method may reduce the probability of triggering the dendritic spikes without significantly affecting the spine signals. Note that, the somatic tuning was measured before photoinhibition of the soma activity, so the measurement of the somatic tuning was not affected by photoinhibition.

Threshold model at the soma to predict somatic tuning

Previous studies (Chen et al., 2013 [↗](#); Wilson et al., 2016 [↗](#); Scholl et al., 2021 [↗](#)) and our study showed that the output tuning was narrower than the total input tuning predicted by simple summation of the inputs. It has also been reported that the tuning curve from the membrane potential is broader than that from soma spiking (Troyer et al., 1998 [↗](#); Finn et al., 2007 [↗](#); Lien & Scanziani, 2013 [↗](#)). To explain the sharp tuning of the soma in V1 neurons, a spike-threshold model has been proposed in which only the preferred orientation raises the membrane potential above the threshold and triggers an action potential in the soma (Priebe & Ferster, 2008 [↗](#)). In our model, we introduced an analogous spike-threshold model for V1 neurons. We assumed that the number of active spines corresponds to the amplitude of the excitatory postsynaptic potential (EPSP) that propagates to the soma and contributes to the level of the soma membrane potential level. We hypothesized that the somatic action potential is evoked only when the number of active spines is above a certain threshold. By applying the threshold model, the tuning of the somatic activity to visual stimulation was sharpened and more accurately predicted, suggesting the importance of thresholding for the output tuning at the soma.

The combined model is better for predicting somatic tuning from synaptic inputs

Previous model study suggested the two-layer input-output function of the pyramidal neuron, that is, synaptic input summation at the dendrite occurs in the first layer and thresholded final response at the soma is produced in the second layer (Poirazi et al., 2003 [↗](#)). This model has been experimentally tested and revealed that combination of dendritic nonlinearity and spike thresholding contribute to the sharpness of orientation tuning in the ferret visual cortex (Wilson et al., 2016 [↗](#)). Therefore, we tested this model using our experimental data if we see further improvement of tuning also in the mice.

The combination of cluster and threshold models improved the prediction of the somatic tuning better than either the individual model. Two steps of integration finally sculps to the sharply tuned output signal. By dividing information processing into two compartments, the soma and the dendrites, neurons may acquire a greater computational capacity than either the cluster or threshold model of integration. Our data are consistent with the previous findings in ferrets (Wilson et al., 2016 [↗](#)) and this two-step sharpening of the output signal may be a common mechanism across species.

Effects of inhibitory inputs on soma output tuning

Excitatory neurons in V1 not only receive excitatory inputs from other excitatory neurons, but also receive inhibitory inputs from interneurons. One of the potential roles of inhibitory inputs for the orientation tuning is to sharpen the soma output of excitatory neurons (Priebe and Ferster, 2008 [↗](#); Liu et al., 2011 [↗](#); Hansel and Vreeswijk, 2012 [↗](#)). The tuning of inhibitory neurons has been shown to be mostly broad for orientation (Sohya et al., 2007 [↗](#); Niell and Stryker, 2008 [↗](#); Kerlin et al., 2010 [↗](#)). Such broad hyperpolarizing potentials can raise the overall excitatory threshold for the soma spiking activity and unselectively suppress the responses at all orientations. Hence, in our model, inhibitory effects are equivalent to increasing the threshold and taken into account in the threshold model.

Role of apical and basal dendritic inputs

Apical and basal dendrites receive inputs from different sources and differ in the density of some types of ion channels (Stuart & Spruston, 2015 [↗](#)), suggesting their different roles in information processing (Spruston, 2008 [↗](#)). In the present study, we recorded only the basal dendrites of layer 2/3 neurons in V1, but previous studies showed that the clustering of spines with similar

orientation preference was not significantly different between apical and basal dendrites in ferret V1 (Wilson et al., 2016 [↗](#)) and the removal of apical dendrite does not alter orientation-tuning (Park et al., 2019). On the other hand, a close relationship between soma firing and activity in the basal dendrite, but not in the apical tuft has been reported (Hill et al., 2013 [↗](#)). Indeed, in our study, soma tuning was estimated only from synaptic inputs on basal dendrites. Regarding the role of apical dendrites, several *in vivo* studies have suggested that synaptic inputs to apical dendrites may play an important role in synaptic plasticity (Cichon & Gan, 2015 [↗](#)), modulation of sensory information (Smith et al., 2013 [↗](#); Palmer et al., 2014 [↗](#)) or reception of surround information in the visual field (Roth et al., 2016 [↗](#)).

Future applications

To understand how neural circuits work, it is important to understand not only the cell types and their connectivity in neural circuits, but also the cell type-specific computation. Cortical neurons can be divided into two types: excitatory and inhibitory neurons. In inhibitory neurons, similar to the excitatory neurons, action potentials initiated in the soma also propagate back into the dendrites (Kaiser et al., 2001 [↗](#)). Because inhibitory neurons generally lack spines and excitatory inputs are formed directly on the shaft (Harris & Shepherd, 2015 [↗](#)), calcium signals derived from BAP are completely mixed with the signals of the shaft synapse, the previous regression and subtraction method (Chen et al., 2013 [↗](#); Wilson et al., 2016 [↗](#); Iacaruso et al., 2017 [↗](#); Scholl et al., 2021 [↗](#)) would be difficult to apply to this type of synaptic input. To our knowledge, only one study has reported the *in vivo* recording of synaptic inputs from inhibitory neurons whose cell bodies do not fire to the visual stimulation (Chen et al., 2013 [↗](#)), and synaptic imaging of inhibitory neurons whose cell bodies do fire has not been attempted because of this difficulty. Therefore, our novel method for recording synaptic activity without interference from BAP signals will be particularly useful for the synaptic imaging of inhibitory neurons.

Previous methods for suppressing BAP (Jia et al., 2010 [↗](#); Chen et al., 2011 [↗](#); Levy et al., 2012 [↗](#)) have not been applicable for repeatable imaging over days; our new method is the first to allow easy and repeatable imaging of synaptic inputs over days *in vivo*. Thus, this new method will contribute to the broader field of synaptic plasticity, which studies studying the dynamic changes in location and activity of synaptic inputs, such as pruning of synaptic inputs during development, pathological alterations of synaptic inputs in the psychiatric disorders and the reorganization of synaptic inputs during learning.

Materials and methods

Animals

C57BL/6 wild-type mice or Thy1-GCaMP6s transgenic mice (GP4.3) (2-3 months old, male) were used for all experiments. C57BL/6 mice were purchased from SLC (Hamamatsu, Japan). Thy1-GCaMP6s mice (stock #024275) were purchased from Jackson Laboratory (Bar Harbor, ME, USA). All mice were maintained in the animal facility at the University of Tokyo and housed 2-3 mice per cage in a temperature-controlled animal room with a 12 h/12 h light/dark cycle. All procedures were conducted in accordance with the protocols approved by the University of Tokyo Animal Care and Use Committee (approval number: H22-104, H22-111).

Local and sparse expression of GCaMP6s and SwiChR++ in V1 layer 2/3 neurons

The genetically encoded calcium indicator GCaMP6s and optogenetic protein SwiChR++ were locally and sparsely expressed using the AAV infection method. A mixture of AAV2/1-hSyn-Cre and AAV2/1-hSyn-flex-GCaMP6s was used for sparse GCaMP6s expression ($\sim 1 \times 10^8$ and $\sim 1 \times 10^{13}$ genome copies/mL, respectively, purchased from the addgene) and AAV2/1-CaMKIIa-mCherry-P2A-

Kv2.1-SwiChR++ for SwiChR++ expression ($\sim 1 \times 10^{13}$ genome copies/mL, originally reconstructed from the AAV vector purchased from the Vector Core of Stanford University). The reconstructed vector for SwiChR++ expression contains mCherry to label SwiChR++-expressing neurons and a Kv2.1 fragment to localize SwiChR++ in the soma to prevent excess hyperpolarization of dendrites. Mice were anesthetized with isoflurane (1.5% vaporized in air) and fixed on stereotaxic frames. The skin was incised at the midline, and the periosteum was removed from the skull. A small craniotomy was performed just above V1 at selected stereotaxic coordinates (-3.5 mm from the bregma, 3.0 mm lateral to the midline), and a glass pipette filled with AAV was inserted (0.4 mm depth from the pia). AAV was injected under pressure (500 nL using a Nanoject III, Drummond Scientific, Broomall, PA, USA). The infection area is typically defined as a 400–700 μm diameter from the injection site. Imaging was performed two weeks after AAV infection.

Imaging

For the imaging of spines and soma of V1 neurons, a craniotomy (4 mm diameter) was performed over V1. The dura mater was removed, and a cranial window was constructed by sealing the opening with a cover slip (No.1, 6.5 mm diameter, Matsunami, Japan). Mice were anesthetized with isoflurane (1.0–2.0%) during surgery. Two-photon calcium imaging of spines and soma was performed under light anesthesia (0.2% isoflurane under sedation with 2.5 mg/kg chlorprothixene). For the spine imaging, neurons that expressed both GCaMP6s and mCherry were selected for imaging. First, imaging of the soma was performed to record the somatic tuning before photoinhibition of somatic activity, and then somatic activity was suppressed by light stimulation and spine imaging was performed. In experiments to examine the effects of light suppression on surrounding cells or spines before and after light suppression, the activity of the cells or spines was first imaged before photoinhibition and then a target cell activity was suppressed by light stimulation and the activity of the same cells or spines was imaged and compared. Imaging was conducted using a two-photon microscope (FVMPE-RS, Olympus, Tokyo, Japan) with a 25 $\times$ objective lens (XPLN25XWMP2, NA = 1.05, Olympus, Tokyo, Japan) at a wavelength of 920 nm (InSight DS, Spectra Physics, Milpitas, CA, USA). Images were obtained in a 2-D plane at 1 Hz (Galvo scanning; for BAP inhibited method) or 15 Hz (resonant scanning; for BAP subtracted method) (Chen et al., 2013 [DOI](#)). The image size was 512 $\times$ 512 pixels (the resolution was 0.125 μm /pixel for spines and 0.5 μm /pixel or 1.0 μm /pixel for soma). The average laser power directed at the sample was modulated between 10 and 40 mW, depending on the imaging depth. To correct the refractive index mismatch in the brain tissue, we carefully adjusted the axial position of the objective lens to obtain the maximum signal intensity.

Visual stimulations were presented on a 32-inch LCD display (ME32B, Samsung) using PsychoPy2 (Peirce, 2008 [DOI](#)). Orientation selectivity was investigated by square-wave drifting gratings in eight directions (45° apart, spatial frequency = 0.04 cpd and temporal frequency = 2 Hz). These eight patterns were presented for ~ 4 s each (4 frames) interspersed with blank (uniform) gray stimuli of either the same duration or twice the duration. The stimuli were presented 10 times.

Optogenetic inhibition of soma activity

Optogenetic inhibition of soma activity was performed by directing a focused visible laser (wavelength: 458 nm, beam diameter: 0.53 μm) through a small round region of interest (ROI) on the soma through an objective lens by Galvano mirror raster scanning (scan size: 20 $\times$ 20 pixels (0.5 μm /pixel), scan speed: 100 ms/frame). The laser power was 50 μW after the objective lens. The duration of the laser stimulation was 100 s. The photoinhibition of somatic activity typically lasted for ~ 60 min, and somatic activity was repeatedly inhibited before recovery from inhibition during the recording of spine activity.

Data analysis

All analyses were performed using custom-written programs in MATLAB (MathWorks, Natick, MA, USA). The data was processed between experimental groups in the same manner using the same computer code; thus, randomization and blinding were not necessary for data analysis. We did not perform experiments blindly. To obtain an orientation map, calcium signal changes were calculated for all pixels, and each pixel was colored according to the response to the orientation stimulation [hue: preferred orientation; lightness: response magnitude; saturation: OSI].

The spines and soma were detected manually using a template-matching algorithm with convolution mask images. The centroids of the spines and soma were determined from a mask image of these structures. Time courses of fluorescent change were extracted by averaging a circle around the centroid (spines, 0.18 μm radius; soma, 2.5 μm radius).

To estimate the out-of-focus signal around individual spines or soma, we created ring-shaped masks with pixels within 10 pixels (spines, 1.25 μm ; soma, 5 μm) from their edge, excluding pixels less than 4 pixels (spines, 0.5 μm ; soma, 2 μm) from the edge of the spines or soma. The out-of-focus signal obtained from these masks was subtracted from the fluorescence signal of the spines and soma (contamination ratio = 0.3) (Kerlin et al., 2010 [DOI](#)).

The orientation selectivity was calculated from the corrected time courses. Visually evoked fluorescent changes were calculated as the change in fluorescence normalized to the baseline fluorescence ($\Delta F/F$). Baseline fluorescence was obtained from the average of the last two frames during the blank periods. The p value for responsiveness ($p(\text{resp})$) was obtained from ANOVA across the blank and stimulus periods, and the p value for selectivity was obtained from ANOVA across stimulus periods.

The preferred orientation was calculated using vector averaging (Swindale, 1998 [DOI](#)), defined by the following equations: $a = \sum R_i \times \cos(2\theta_i)$; $b = \sum R_i \times \sin(2\theta_i)$, $\theta_{\text{pref}} = 0.5 \arctan(b/a)$. R_i is the response to the i^{th} direction θ_i (eight directions, 45° apart, spanning $0-315$ degrees). θ_{pref} is the preferred orientation. The orientation selectivity index (OSI) was calculated using the following formula: $(R_{\text{pref}} - R_{\text{ortho}}) / (R_{\text{pref}} + R_{\text{ortho}})$, where R_{pref} is the response to the preferred orientation and R_{ortho} is the response to the orientation orthogonal to the preferred orientation. Spines or neurons were considered responsive when they met the following criteria: the p value for responsiveness was less than 0.01, and the maximum response was more than 3%. Among responsive spines or neurons, sharply orientation-selective spines were defined when they met the following criteria: the p value for selectiveness was less than 0.01, and the OSI was more than 0.3.

The preferred direction was calculated using a Gaussian fitting method. A tuning curve was fitted with a sum of two circular Gaussian functions (von Mises distribution), and the peak of the fitting curve was considered the preferred direction. As an index of direction selectivity, DSI was calculated using the following formula: $\text{DSI} = (R_{\text{pref}} - R_{\text{opp}}) / (R_{\text{pref}} + R_{\text{opp}})$, where R_{pref} is the response to the preferred direction and R_{opp} is the response to the opposite of the preferred direction. The responsive boutons or neurons were defined using the same criteria as for the orientation analysis. Among responsive boutons or neurons, sharply direction-selective boutons were defined as those that met the following criteria: the p value for selectiveness was less than 0.001, and the DSI was more than 0.3.

Spine activities were calculated by subtracting the scaled dendritic signal, as previously reported¹⁰. Briefly, dendritic shafts ($\sim 20 \mu\text{m}$ in length) excluding spines were extracted and $\Delta F/F$ ($\Delta F/F_{\text{dendrite}}$) was calculated. Individual spine signals ($\Delta F/F_{\text{spine}}$) were plotted against $\Delta F/F_{\text{dendrite}}$, revealing two components: a dendrite signal component and a spine signal component. To remove the bAP-related dendrite signal component from the spine signals, the

scale factor for the dendrite signal component was determined, and the spine-specific signal was calculated as follows: $\Delta F/F_{\text{spine_specific}} = \Delta F/F_{\text{spine}} - \alpha \Delta F/F_{\text{dendrite}}$. α was determined using robust regression (MATLAB function 'robustfit.m') of $\Delta F/F_{\text{spine}}$ vs. $\Delta F/F_{\text{dendrite}}$ (the slope of the fitted line in Figure S10).

To compare the experimental results with randomness, we generated 1,000 new maps of functional spine input maps for each experiment. These randomized maps contained the same proportion of orientation tuned spines and orientation tuned spines were repositioned randomly in each analysis, while preserving the original spine positions.

The correlation index (CI) between the actual and estimated tuning curve of the soma was calculated as follows:

$$CI = A / \sqrt{(B \times C)},$$

$$A = \sum (\text{actual tuning curve} - \text{mean (actual tuning curve)}) \times (\text{estimated tuning curve} - \text{mean (estimated tuning curve)})$$

$$B = \sum (\text{actual tuning curve} - \text{mean (actual tuning curve)})^2,$$

$$C = \sum (\text{estimated tuning curve} - \text{mean (estimated tuning curve)})^2.$$

Estimation of tuning curve of soma activity by weighting

First, we divided the spines into two types: one was visually responsive (spine activity = 1) and the other was visually unresponsive (spine activity = 0). The effect of visually responding spines on soma tuning was weighted by multiplying either signal change ($\Delta F/F$) or distance between the soma and the given spine (x) by the spine activity value (either 0 or 1). When using signal change as a weighting factor, we estimated the tuning curve of the soma activity as follows:

$$\text{Tuning curve of soma activity} = \sum (\Delta F/F) \times (\text{spine activity})$$

($\Delta F/F$) is the signal change of the spine to visual stimulation in one of 8 directions. Spine activity at one of 8 directions is assigned 0 or 1 (0: not responsive, 1: responsive). When using the distance between the soma and a given spine as a weighting factor, we estimated the tuning curve of soma activity as follows:

$$\text{Tuning curve of soma activity} = \sum (\exp(-x/L)) \times (\text{spine activity})$$

x is the distance between the soma and a given spine and L is the length constant. L was fixed at 200 μm in [Figure 6e](#) and varied between 100 μm , 200 μm , and ∞ in [Figure S14a](#) and [13b](#).

Spine activity at one of 8 directions is assigned 0 or 1 (0: not responsive, 1: responsive).

Reconstruction of functional synaptic input map

To perform quantitative analyses of the functional geometric organization and synaptic integration model, we reconstructed the functional synaptic input map. Before starting the functional imaging of spines, we took 3-D images of recorded neurons (0.5 $\mu\text{m}/\text{pixel}$) with a 1 μm z-step and reconstructed the anatomical 3-D image. The locations of spines in the functional 2-D images were reconstructed on anatomical 3-D images by aligning the functional 2-D images with the anatomical 3-D image. The coordinates of the spines were defined by setting the coordinates of the center gravity of soma as (0,0,0) and the reconstructed 3-D functional synaptic input map. The

distance between the soma and a given spine or between two given spines was calculated as the Euclidean distance using the 3-D functional synaptic input map. The average length of the dendritic branches in our recorded neurons was $24.3 \pm 1.1 \mu\text{m}$ (6 neurons, 214 branches).

Correlation analysis between two spine activities

To calculate the correlation of the two spine activities, we applied the shuffle-corrected cross-correlation method. First, we calculated trial-averaged time-course data for each stimulus as the shuffle-predictor of visual response. The shuffle-corrected time-course data were obtained by subtracting the shuffle-predictor from the original time-course data for each stimulus. The shuffle-corrected cross-correlogram was obtained as a cross-correlogram between shuffle-corrected time courses. This procedure is equivalent to repeating the shuffle correction for every possible shuffle. The correlation coefficients (values of the shuffle-corrected cross-correlogram at time zero) were calculated between the two spines.

Analysis for the relationship between two forms of functional clustering

To analyze the relationship between the clustering at the spine-pair level and at the dendritic branch level, we calculated the correlation-coefficient between the number of similarly responded spine pairs and the number of similarly responded spines on each dendritic branch. Then, we generated 1,000 new synaptic input maps (randomized maps) for each experiment. Randomization was done either within each dendritic branch or across cell. These randomized maps contained the same proportion of orientation, and the orientation-selectivity were repositioned randomly in each analysis, while preserving the original 3-D spine positions. From these randomly repositioned maps, we calculated the correlation-coefficient as described above and statistically compared the correlation-coefficient values from the experimental map and two different randomized maps.

Cluster model analysis

First, we counted the number of visually responding spines in each direction on individual dendritic branches. We set the variable threshold (1–10) for the number of spines in each direction of visual stimulation. If the number of spines exceeded the threshold, we regarded these spines as contributors to soma activity. We repeated this analysis on all the dendritic branches and summed the number of responding spines for each direction of visual stimulation. Finally, we estimated the tuning curve of soma activity from the summed number of spines in each direction of visual stimulation.

Threshold model analysis

In the threshold model, we regarded all the visually responsive spines as contributors to the soma activity, but somatic spikes occurred only when the number of summed responding spines at the soma was above the threshold number of spines. We varied the threshold, and the tuning curve of soma activity was estimated by subtracting the threshold number of spines from the summed number of spines.

Immunohistochemistry

AAV infected mouse (two weeks after infection) was fixed by perfusing 4 % paraformaldehyde solution via vasculature system through heart. Brain was removed and post-fixed in the same fixative solution for overnight at 4 degrees. Postfixed brain was cryopreserved by dehydration with sucrose solution, starting at 10% concentration and gradually increasing to 20% and 30%. Coronal slice (50 μm thickness) from primary visual cortex was prepared by a cryostat (CM1520, Leica). Brain sections were run through an immunohistochemistry protocol in order to reveal the hemagglutinin (HA) tag fused to the SwiChR++ as follows. (1) blocking 1h with 5% goat serum + 2.5% Bovine Serum Albumin + 0.1% Triton X-100 in 0.01M PBS; (2) reacted with primary antibody

(High affinity anti-HA rat monoclonal antibody (clone3F10), catalogue# 11867423001, Roche), 1:200, 24h at 4 C; (3) washed with PBS at room temperature, 3 times, 15 min for each; (4) reacted with secondary antibody tagged with Alexa Fluor 488 (goat anti-rat IgG, product number A11006, Invitrogen), 1:200, 2-3h at room temperature; (5) washed with PBS at room temperature, 3 times, 15 min for each. Brain sections were mounted with aqueous mounting solution (ProLong Gold, Thermofisher) and analyzed by confocal microscopy (A1R, Nikon).

Statistical analysis

All data are presented as the mean $\pm$ standard error of the mean (SEM) unless stated otherwise. No statistical methods were used to pre-determine sample sizes, but our sample sizes are similar to those generally employed in the field. A one-sample t-test was used to compare a single sample mean to a null hypothesis value. A paired-sample t-test was used to compare between paired observations. A two-sided Mann-Whitney U-test or an unpaired-sample t-test was used to compare two independent groups. An ANOVA and Bonferroni-corrected post hoc test were performed when more than two groups were compared.

Acknowledgements

We thank Dr. M. Uemura and Virus Vector Core (Prof. Kobayashi's Group) of Brain/MINDS (Brain Mapping by Integrated Neurotechnologies for Disease Studies) for the production of AAV; A. Hayashi, Y. Kato, M. Taki, T. Inoue, Y. Sono, A. Honda for animal care and genotyping; all of the members of Ohki laboratory for support and discussion; The Genetically-Encoded Neuronal Indicator and Effector (GENIE) Project for providing GCaMP6s. Prof. K. Deisseroth for providing SwiChR++.

This work was supported by grants from Brain/Minds from AMED (Grant numbers, JP17dm0207048, JP21dm0207014 to K.O.), Brain/MINDS 2.0 from AMED (JP23wm0625001 to K.O.), JST-CREST (JPMJCR22P1 to K.O.), Institute for AI and Beyond to K.O., JSPS KAKENHI (Grant numbers 17H03540, 20H03336, 21H05165, 23H02572, 24H02331 to SK, 19H05642, 20H05917 to KO), AMED (Grant number JP21wm0525013 to SK), The Mitsubishi Foundation to SK.

Additional information

Author contributions

S.K. and K.O. designed the research. S.K. and K.K. performed the experiments and analyzed the data. S.K. and K.O. wrote the manuscript.

Data and code availability

The data that support the findings of this study and all custom code used in this study are available from the corresponding author upon reasonable request.

Competing financial interests

The authors declare no competing financial interest.

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Reviewer #1 (Public review):

Summary:

In this manuscript, Kondo et al. developed a method to suppress somatic action potentials while recording spine calcium signals using two-photon imaging in the L2/3 visual cortex in response to visual stimuli. The authors identified different patterns of dendritic spine activation by visual stimuli and analyzed how the different patterns of spine responses may contribute to somatic visual responses. Their analysis results suggest that spines on dendrites with a clustered arrangement can potentially generate sharply tuned output.

Strengths:

This is an interesting study addressing a standing question of how previously reported pepper-and-salt-like distributed sensory inputs on individual spines may give rise to somatic sensory selectivity. The method of somatic inhibition to prevent bAPs appears new and effective. The measurements of spine activity are carefully done. The finding that a small number of spines located in the same branch with similar tuning properties would predict the somatic tuning is consistent with local dendritic nonlinear integration mechanisms.

Weaknesses:

(1) The demonstration of the effectiveness of soma-specific inhibition is inadequate. Figure 1 only provides a single example trace showing the inhibition of somatic visual responses. The authors should provide statistical analysis over grouped data. For the effect of soma-specific inhibition on spine activity, the authors provided mostly negative results, lacking effects on spine responses for both soma inhibition and bAP subtraction. This is confusing. One possible explanation is that bAPs normally have little influence on spine activity. However, this would conflict with the known fact that somatic APs can easily invade spines in L2/3 neurons (e.g., Chen et al., Nature 2011). Another possibility is that under the current experimental conditions, somatic APs were rarely evoked by the visual stimulus. The authors should also rule out the possibility that the spines they imaged are from different neurons than the ones with somatic inhibition. The authors may consider identifying those cases where somatic APs have a significant impact on spine activity or spine tuning and show how bAP inhibition influences the dendritic and spine responses.

(2) Figure 4 shows that the proportion of spines with a preferred orientation similar to the soma ($\Delta\text{Ori} \{\text{less than or equal to } 30\{\text{degree sign}\}\}$) was 60%, which is surprisingly high. It is intriguing that without somatic AP invasion, there could be such a high degree of similarity between spine activity and somatic tuning. What is the ratio without soma inhibition? One could reason that with bAP invasion, there should be even more spines showing visual responses similar to those of the soma. Moreover, with such a high proportion of spines showing similar sensory tuning to the soma, it is inevitable that many branches contain more spines with similar tuning as the soma, exhibiting an apparent branch-specific clustering.

While such apparent clustering may well predict somatic tuning, it primarily reflects a correlational relationship rather than a causal synaptic integration mechanism.

(3) There has been extensive work studying how the integration of spine activity or sub-branch activity gives rise to somatic output. The proposed main contribution of this study is to use an improved method to inhibit somatic activity in order to more confidently measure spine-specific activity and examine the integration mechanisms. However, the results showed that the measured spine-specific activity under soma inhibition was not significantly different from that measured under normal conditions (see point 1). It becomes unclear how this new method contributes to obtaining new insights into the synaptic integration mechanism.

(4) Figure 6 shows how the tuning similarity between spines depends on the distance between them. It is unclear what new information was acquired regarding the functional clustering of spines. This result can be largely explained by the overall higher proportion of similarly tuned spines (60%) compared to the soma's preferred orientations. Moreover, the authors did not demonstrate how such clustering may contribute to nonlinear synaptic integration.

(5) The results shown in Figure 7 can again be largely explained by the static property of a higher proportion of spines tuned similarly to the soma. These results do not reveal any active dendritic integration mechanisms.

<https://doi.org/10.7554/eLife.104334.1.sa1>

Reviewer #2 (Public review):

Summary:

The paper from Kondo et al., addresses how the functional organization of synaptic inputs in 2/3 pyramidal neurons contributes to their output firing. Expressing GCaMP6s to monitor calcium activity and the bi-stable inhibitory opsin SwiChR++ to inhibit the somatic activity of the imaged neurons, the authors were able to image up to ~5700 spines in basal dendrites from 6 neurons. Mapping the functional responses of such a large number of dendritic spines and relating it to the output firing of the parent neuron is a remarkable feat. The authors studied the clustering of similarly tuned spines within individual dendrites and found that while some dendrites are similarly tuned to the same orientation of the parent neuron, other dendrites exhibit tuning to other orientations and moreover a significant proportion of dendrites exhibit no tuning. Modelling work suggests that the clustering of spines in a small proportion of dendrites should suffice to give rise to the tuning of the parent cell.

Strengths:

(1) Removal of the potential confound of somatic firing via optogenetic inhibition is convincing and validates a useful tool for the neuroscientific community. As discussed by the authors the tool would be most valuable for the study of excitatory inputs in inhibitory neurons.

(2) The comparison of optogenetic inhibition of somatic responses and isolation of spine-specific signals using the removal of backpropagating action potential by robust regression is an important control and constitutes an important affirmation of previously published work.

(3) The large dataset size provides enough statistical power to test for clustering of similarly tuned spines in basal dendrites.

(4) The study provides a useful replication of previously published results.

(5) Modelling work in the study shows that as in the ferret visual cortex (Wilson et al., 2016), a combination of dendritic nonlinearity and spike thresholding contribute to the sharpness of orientation tuning in the mouse visual cortex.

Weaknesses:

(1) One of the main conclusions of the study, the classification of dendrites according to the presence or absence of visual responses, lacks quantification.

(2) Some of the statistics employed in combination with shuffling controls are not adequate.

(3) All the neurons imaged are very highly tuned (with a very high orientation selectivity index (OSI)). The performance of the models is evaluated by the correlation coefficient between the predicted and the measured somatic tuning curve. The high OSI of the neurons reduces the sensitivity of the evaluation of the models, as it results in extremely high or low correlation coefficients (Figure 8a). It would be important to recapitulate the results from the model for neurons with lower OSI, given that not all L2/3 neurons are so highly tuned.

(4) It is very hard to understand how the modelling results relate to the experimental data, as the definitions of what constitutes a clustered dendrite in the model or in the experimental data are unclear.

<https://doi.org/10.7554/eLife.104334.1.sa0>