Orientation selectivity and the functional clustering of synaptic inputs in primary visual cortex

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The majority of neurons in primary visual cortex are tuned for stimulus orientation, but the factors that account for the range of orientation selectivities exhibited by cortical neurons remain unclear. To address this issue, we used in vivo two-photon calcium imaging to characterize the orientation tuning and spatial arrangement of synaptic inputs to the dendritic spines of individual pyramidal neurons in layer 2/3 of ferret visual cortex. The summed synaptic input to individual neurons reliably predicted the neuron's orientation preference, but did not account for differences in orientation selectivity among neurons. These differences reflected a robust input–output nonlinearity that could not be explained by spike threshold alone and was strongly correlated with the spatial clustering of co-tuned synaptic inputs within the dendritic field. Dendritic branches with more co-tuned synaptic clusters exhibited greater rates of local dendritic calcium events, supporting a prominent role for functional clustering of synaptic inputs in dendritic nonlinearities that shape orientation selectivity.

The selective responses of neurons in visual cortex to the orientation of edges has served as a focal point for elucidating fundamental mechanisms that underlie neural circuit function in cerebral cortex. The majority of neurons in primary visual cortex are orientation tuned, exhibiting the greatest response for the neuron's preferred orientation and lesser responses for other orientations that fall within its tuning bandwidth. In addition to differing in their preferred orientation, individual cortical neurons exhibit considerable variation in selectivity1–3. Differences in selectivity endow cortical neurons with different sensitivities to changes in stimulus orientation4, and recent evidence suggests that this diversity enhances the ability of cortical circuits to encode visual information in natural scenes1.

Despite the prominence of these selectivity differences and their significance for cortical function, the underlying mechanisms that account for diversity in orientation selectivity remain unclear. The natural starting point for probing this issue is to ask whether differences among neurons in orientation selectivity are simply a reflection of differences in the orientation tuning of their excitatory synaptic inputs. Several lines of evidence, direct and indirect, from a range of species indicate that there is a bias for synaptic connections to link cortical neurons with similar orientation preferences1–5. But it is not clear whether the orientation selectivity that a neuron exhibits in its spike discharge can be reliably predicted from the tuning of its synaptic inputs. One line of evidence consistent with this possibility comes from studies in species that have columnar maps of orientation preference. On average, neurons located near pinwheel centers, where adjacent neurons have quite different orientation preferences, exhibit broader orientation tuning than neurons in regions of the map where neighboring neurons exhibit similar preferences9. This variation in selectivity could reflect differences in the range of orientations represented in synaptic inputs derived from neighboring neurons, but a direct assessment of the input–output functions for cortical neurons is lacking.

Beyond potential differences in the tuning of synaptic inputs, cellular nonlinearities that influence neuronal input–output functions are also critical for understanding differences in orientation selectivity. The nonlinearity imposed by spike threshold plays a powerful role in filtering weak subthreshold inputs and sharpening the spike discharge tuning function10. But how much of the differences in selectivity in cortical neurons is attributable to differences in spike threshold remains unclear. Likewise, nonlinearities in the integration of synaptic inputs within dendritic processes could make substantial contributions to differences in orientation selectivity. Synaptic inputs that are sufficiently clustered in space and time can lead to local dendritic events, including NMDA-type glutamate receptor–dependent spikes11–13, and these local dendritic events appear to enhance feature selectivity and responsiveness in visual and somatosensory cortex14–16. While functional clustering of synaptic inputs has been observed in hippocampal neurons in vitro17,18 and in spontaneous activity patterns of inputs to cortical neurons in vivo19, evidence that clustering of functionally similar synaptic inputs contributes to enhanced selectivity in vivo remains elusive19–21.

We used in vivo two photon calcium imaging to characterize the orientation tuning and spatial arrangement of synaptic inputs to dendritic spines of individual pyramidal neurons in layer 2/3 of ferret visual cortex. Differences in the breadth of input tuning and spike threshold alone were insufficient to account for the diversity of orientation selectivity displayed by layer 2/3 neurons. Instead, we found evidence for orientation-specific clustering of synaptic inputs that correlates with the likelihood of local dendritic calcium events and predicts the degree of orientation selectivity.

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RESULTS
Functional dendritic spine imaging in ferret visual cortex
We assessed the spatial organization of synaptic inputs in layer 2/3 pyramidal neurons using in vivo two-photon imaging of calcium dynamics following AAV expression of the genetically encoded sensor GCaMP6s in the ferret visual cortex. Sparse labeling (Online Methods) allowed us to trace dendrites and resolve individual dendritic spines without background fluorescence contamination. Calcium responses from soma and dendritic segments (field of view: 42 × 42 µm) were imaged serially during presentation of visual stimuli (Fig. 1a–d). Visual stimuli consisted of drifting gratings of different orientations (Online Methods). Using a computational procedure to isolate synaptic calcium fluorescence from dendritic shaft signals, we extracted the synaptic responses of single dendritic spines (Supplementary Figs. 1 and 2). We imaged a total of 2,184 spines within the dendritic fields of nine neurons from eight animals (median = 245, range = 178–299 per neuron; Fig. 1e). Of the 2,184 spines imaged, we included for further analysis 1,876 (85.9%) of spines whose synaptic calcium transients were deemed separable from dendritic signals. Of these spines, 1,175 (62.6%) showed mean response at the preferred stimulus greater than 10% ΔF/ΔF with SNR >1. Of these spines, 836 (71.1%) could be fit with significant Gaussian tuning curves (r > 0.7, P < 0.05, Pearson’s correlation coefficient). A median of 105 spines were included per neuron, with a range of 58–118.

Individual spines exhibited strong orientation selectivity (Fig. 1b), and their orientation tuning bandwidth was similar to the somatic tuning bandwidth (Wilcoxon rank-sum, median spine tuning bandwidth = 11.2°, interquartile range (IQR) 8.5° to 17.8°, median somatic bandwidth = 11.3°, IQR 8.7° to 16.1°; P = 0.88, n = 9 cells and n = 836 spines). To compare the orientation tuning of individual neurons with their synaptic input, we summed the fluorescence responses of all active spines on each neuron (Fig. 2a,b). The orientation preference of the summed spine inputs strongly predicted somatic orientation preference (Fig. 2c; Wilcoxon signed-rank, signed-rank = 25, P = 0.82).

Summed spine orientation selectivity was consistently lower than somatic selectivity, as measured by 1 – circular variance (Fig. 2d, spine median = 0.24, soma median = 0.83, n = 9, P = 0.0039, Wilcoxon signed-rank), but we found orientation selectivity to vary broadly in both somata (0.58 to 0.97) and summed spine inputs (0.16 to 0.38).

Relationship of spine tuning to orientation preference map
As a first step in considering the factors that could account for the variation in selectivity among neurons, we asked whether the degree of orientation selectivity was correlated with the neuron’s cortical location. The ferret visual cortex, like that of many other non-rodent species, exhibits an orderly columnar map of orientation preference. Previous studies suggest that neurons in regions of the cortex where orientation preference exhibits a high rate of change (HRoC) (pinwheel centers) are less selective for orientation than neurons in regions where orientation preference exhibits a low rate of change (LRoC). We aligned the location of each cell body and dendritic field with the intrinsic signal orientation preference map using control point registration and an affine transformation (Fig. 3a,b and Supplementary Fig. 3). Somatic orientation preference of individual neurons was consistent with the preference predicted by somatic location within the orientation map (Fig. 3c; Wilcoxon signed-rank, signed rank = 27, P = 0.65, n = 9). However, we found no strong relationship between somatic or summed spine orientation selectivity and position within the orientation preference map (Fig. 3d; soma: r = −0.027, P = 0.95, n = 9; spines: r = 0.65, P = 0.06, n = 9; slope < 0.001). Moreover, after calculating the orientation preference of each spine relative to the soma, we found no significant difference in the diversity of spine inputs onto individual neurons in HRoC areas vs. LRoC areas (Fig. 3e; Wilcoxon rank-sum, P = 0.61, n = 366 spines on cells in LRoC regions and n = 470 spines on cells in HRoC regions). We found that individual spine orientation preferences corresponded weakly with the orientation preference (Ori) map (median ΔOri between spines and map 37.9° with IQR 41.2°, n = 836; Supplementary Fig. 4).

Figure 1. Response properties of orientation-selective synaptic inputs to neurons in the ferret visual cortex. (a) Average somatic and spine responses to visual stimuli; mean is black and s.e.m. is gray. (b) Example orientation tuning curves. Black bars, mean ± s.e.m.; blue lines, fits. (c) Nuclear-excluded soma; scale bar, 5 µm. (d) Dendritic segment with spines whose tuning curves are depicted in b; scale bar, 5 µm. (e) Single neuron (triangle) with all serially imaged orientation-selective spines (circles) overlaid on intrinsic signal map and colored by orientation preference; scale bar, 50 µm.
Nonlinear synaptic integration in visual cortex

Variation in somatic selectivity might be accounted for simply by differences in the selectivity of summed spine inputs. However, across our sample of neurons, the selectivity of summed spine inputs was not significantly correlated with somatic orientation selectivity ($r = 0.45, P = 0.22, n = 9$ neurons, Pearson’s correlation coefficient). Moreover, after summing the spine inputs and applying a spike threshold, we found considerable variation in the input–output functions for each neuron, (Fig. 4a,b, Online Methods and Supplementary Fig. 5). Somatic responses in some neurons were well predicted by synaptic inputs (quasi-linear input–output functions), while others displayed sharpened somatic selectivity relative to synaptic inputs (nonlinear input–output functions) (Fig. 4b,c). The degree to which a linear summation and somatic threshold could reconcile differences between summed spine and somatic orientation tuning was correlated with the neuron’s somatic orientation selectivity, such that neurons with greater orientation selectivity exhibited a more nonlinear input–output function ($r = 0.79, P = 0.001$, Pearson’s correlation coefficient, $n = 9$, Fig. 4c).

One obvious source of this variation in the degree of nonlinearity could be differences in absolute spike threshold: a higher spike threshold would limit evoked spikes to a narrower range of subthreshold inputs, thereby forming the basis for a nonlinear input–output relationship. If this were the case, we would expect a systematic relationship between spike threshold and orientation selectivity. We explored this possibility by performing in vivo whole-cell recordings (Fig. 4d–f, $n = 16$ cells from six animals) to measure subthreshold membrane potential orientation selectivity, spike threshold, and spiking orientation selectivity. Subthreshold membrane potential and spiking both showed robust responses to visual stimuli (Fig. 4e,f), but membrane potential was considerably less orientation selective than spiking (Wilcoxon signed-rank, $P = 5.2 \times 10^{-4}$, Supplementary Fig. 6), consistent with the role of spike threshold in sharpening orientation tuning.

To determine whether variation among neurons’ spike threshold could account for differences in selectivity, we measured the distance from resting membrane potential to spike threshold and found that spike threshold was not correlated with spiking orientation selectivity.

Figure 3 Location in orientation preference map reliably predicts somatic orientation preference but not somatic or synaptic input selectivity. (a,b) Dendritic spine (circles) and somatic (triangles) orientation preference overlaid onto orientation preference map in low and high rate of change areas; scale bar, 50 μm. (c) Somatic orientation preference is strongly correlated with the underlying intrinsic signal map. (d) Distance from high gradient region does not reliably predict somatic (triangles) or summed spine (circles) orientation selectivity. (e) The distribution of spine orientation preference relative to somatic orientation preference is not significantly different between cells in regions with low rates of change versus cells in regions with high rates of change. See main text for $P$ value.
Figure 4  Nonlinear synaptic integration in visual cortical neurons. (a) Generation of input-output (I/O) transfer function. (b) Input-output transfer functions for all cells, colored by orientation selectivity. (c) Somatic orientation selectivity is correlated with nonlinearity in the input–output functions. (d) Top, single-trial membrane potential with spikes included; bottom, average membrane potential after removing spikes. (e,f) Membrane voltage ($V_m$) and spiking tuning curves for whole-cell recordings peak-aligned to 67.5°; gray lines are individual neurons and black lines are population average. (g) Distance to threshold was measured as distance from resting potential to the kink in the AP waveform for 10 spikes per neuron. (h) Distance to threshold is not correlated with spiking orientation selectivity; error bars, ±s.e.m.

$(r = 0.31, P = 0.24, n = 16, \text{Fig. 4g,h})$. Thus, while spike threshold clearly enhances feature selectivity by filtering out the unselective component of the summed inputs that reach the soma (Figs. 2a,b and 4e,f), spike threshold alone does not account for the variation in spiking orientation selectivity that we find across neurons. Finally, in comparing the orientation selectivity of somatic calcium signals and spiking responses from whole-cell recordings, we found no significant difference between the distributions of selectivity derived using each technique (two-sample Kolmogorov-Smirnov test, $P = 0.38; n = 9$ cells and $n = 16$ cells, respectively). This finding allows us to exclude the possibility that nonlinearities in GCaMP6s might distort our measures of somatic orientation selectivity.

Functional clustering of synaptic inputs

These observations led us to consider the possibility that variations in input–output functions might be attributable to additional nonlinearities in the dendritic tree. Recent work has implicated NMDA spiking and other nonlinear dendritic mechanisms involving correlated activity of neighboring synaptic inputs$^{11–13}$ in sharpening cortical feature selectivity$^{14–16,29}$. We began by assessing the orientation preference of adjacent spines on branches with at least three active, orientation-tuned spines and found that neighboring spines along dendritic branches ($n = 765$) tended to share similar orientation preferences (Supplementary Fig. 7a). The similarity in the orientation tuning of nearby spines was also evident at the scale of individual dendritic segments (within our 42-µm field of view), quantified by measuring the circular dispersion of spine orientation preferences on these same dendritic branches. Dendritic segments of individual neurons exhibited a broad range of circular dispersions (1.7° to 40.4°; Fig. 5a), and branches from apical ($n = 74$) and basal ($n = 72$) branches showed similar levels of circular dispersion (median dispersions of 20.1° and 20.8° with IQR of 14.3° and 14.6°, respectively, Wilcoxon rank-sum, $P = 0.57$; Supplementary Fig. 7b). But, notably, neurons displaying greater somatic orientation selectivity exhibited dendritic segments with more homogeneous orientation preferences (Fig. 5b and Supplementary Fig. 7c).

To better understand the significance of homogeneous synaptic organization, we measured the coactivation of individual spines on the same dendritic branch. Trial-to-trial correlations of active, orientation-tuned spines on a given dendritic branch were correlated with dendritic branch circular dispersion (Fig. 5c, $r = −0.30, P = 0.0002$, Pearson's correlation coefficient, $n = 146$). Further, branches with the least dispersion (<15°) had significantly greater trial-to-trial correlations than those with heterogeneous spine preferences (circular dispersion >15°) (circular dispersion <15°: median correlation = 0.32, $n = 43$; circular dispersion >15°: median correlation = 0.26, $n = 103$; Wilcoxon rank-sum, $P = 0.0035$). As such, we used this cutoff (circular dispersion <15°) as a working definition for functional synaptic clusters in our data set. The density of functional clusters per millimeter dendrite on single neurons was highly correlated with the neuron's somatic orientation selectivity ($r = 0.71, P = 0.03$, Pearson's correlation coefficient, $n = 9$, Fig. 5d) and the nonlinearity of the input–output relationship ($r = 0.70 P = 0.036$, Pearson's correlation coefficient, $n = 9$, Fig. 5c). In addition, the orientation preference of spines within functional clusters was more similar to that of the soma than spines outside clusters (branch circular dispersion >30°) (Fig. 5f; Wilcoxon rank-sum, $P = 1.51 \times 10^{-12}$, $n = 219$ spines in clusters with median $\Delta$Ori with soma = 16.9° and $n = 136$ spines not in clusters with median $\Delta$Ori with soma = 35.3°), consistent with a preferential contribution of clustered inputs to somatic orientation tuning.

Dendritic nonlinearities and orientation selectivity

If functional clusters of synaptic inputs influence somatic orientation tuning via dendritic nonlinearities, we would expect to see differences in the functional properties of dendrites with and without functional clusters. To explore this issue, we measured the spatial profile of stimulus-evoked calcium events within dendritic branches. After excluding the possibility that large calcium transients (>10 µm full width at half-maximum (FWHM)) could result from passive diffusion of calcium from the spine to the dendritic branch (Supplementary Fig. 2a), we found that some calcium events propagated almost uniformly throughout the branch, while others manifested as local hotspots in dendritic calcium concentration (Fig. 6a–d and Online Methods). After removing the uniform components of the dendritic signal contributing to dendritic calcium responses (Supplementary

$(r = 0.31, P = 0.24, n = 16, \text{Fig. 4g,h})$. Thus, while spike threshold clearly enhances feature selectivity by filtering out the unselective component of the summed inputs that reach the soma (Figs. 2a,b and 4e,f), spike threshold alone does not account for the variation in spiking orientation selectivity that we find across neurons. Finally, in comparing the orientation selectivity of somatic calcium signals and spiking responses from whole-cell recordings, we found no significant difference between the distributions of selectivity derived using each technique (two-sample Kolmogorov-Smirnov test, $P = 0.38; n = 9$ cells and $n = 16$ cells, respectively). This finding allows us to exclude the possibility that nonlinearities in GCaMP6s might distort our measures of somatic orientation selectivity.

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Our results show that orientation-tuned neurons in layer 2/3 of visual cortex generate well-tuned responses from a broad range of synaptic inputs. Although the summed synaptic input to individual neurons reliably predicted the neuron's orientation preference, it did not account for differences among neurons in orientation selectivity. These differences reflected a robust input–output nonlinearity that could not be explained by spike threshold alone and was strongly correlated with the spatial clustering of co-tuned synaptic inputs within the dendritic field, as has been proposed in previous studies.30–32. Combined with evidence that dendritic branches with co-tuned synaptic clusters exhibited a greater frequency of calcium hotspot activation, these results support a prominent role for functional clustering of synaptic inputs in dendritic nonlinearities that shape orientation selectivity.

The demonstration that a neuron's preferred orientation could be predicted from the sum of its synaptic inputs is consistent with results of a study that examined the orientation tuning of dendritic spines and somata of individual pyramidal neurons in layer 2/3 of the mouse. It is also in agreement with a number of other studies that have demonstrated functionally biased connectivity among neurons with similar feature selectivity.3,33. But our results also reveal just how much the functional specificity in connections deviates from the classic 'like connects with like' description, showing substantial synaptic input from neurons showing substantial synaptic input from neurons that prefer orientations orthogonal to that of the soma. These results are also consistent with the broad tuning of subthreshold potentials revealed by in vivo intracellular recordings,3,14,21,25–27, and they emphasize the critical function of cellular nonlinearities that shape neuronal input–output functions in generating coherent neural representations of stimulus features.

Indeed, our results suggest that the diversity in orientation selectivity that characterizes the responses of cortical neurons rests heavily on...
differences in these input–output nonlinearities. In our sample of neurons, somatic orientation selectivity could not be predicted from the tuning of synaptic inputs, and considerable differences in selectivity were found for neurons that had similar input tuning. At first glance, this may seem inconsistent with the evidence for differences in selectivity between neurons that lie near pinwheel centers and those in the centers of orientation domains, differences that have been ascribed to local inputs from neighboring neurons39. However, while there may be neurons with broader tuning near pinwheel centers, it is also evident that there is considerable diversity in the orientation selectivity of neighboring neurons in all regions of the orientation map24,25. This is consistent with results from the neurons in our sample, where we found little relation between the orientation tuning of the soma and distance to pinwheel centers. In short, our results indicate that the tuning of synaptic inputs and the cellular nonlinearities that shape a given neuron's orientation selectivity cannot be reliably inferred from the neuron's location in the orientation preference map.

Our evidence suggests that nonlinearities driven by the functional clustering of synaptic inputs within the dendritic tree are a major contributor to diversity in orientation selectivity in layer 2/3 neurons. Our conclusions rest on previous studies showing that coincident synaptic inputs can lead to local dendritic events, including NMDA receptor–dependent spikes11–13,35, and that these events enhance feature selectivity and responsiveness in visual and somatosensory cortex14–16. What has been missing from previous studies is in vivo evidence for the clustering of functionally similar synaptic inputs that would be expected to drive dendritic nonlinearities and enhance selectivity19–21. Our results show that the density of functional clusters is significantly correlated with the orientation selectivity of individual neurons and the nonlinearity of their input–output functions. Combined with the demonstration that local dendritic hotspots associated with functional synaptic clusters are almost twice as likely to be activated by stimulation with the preferred orientation, our observations provide a compelling case linking functional synaptic clustering, dendritic nonlinearities, and orientation selectivity. It is likely that this mechanism generalizes to other species and cortical areas that exhibit selective responses.

Methods

Methods and any associated references are available in the online version of the paper.

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

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Farms) aged P21–P23 were anesthetized with ketamine (50 mg/kg, IM) and Flex.GCaMP6s (7.31 × 10^{12} GC ml^{-1}, Penn Vector Core) to sparsely express of visual experience, ferrets were anesthetized with 50 mg kg^{-1} ketamine and 1–3% isoflurane. Atropine (0.2 mg kg^{-1}, SQ) and bupivacaine were administered. Upon completion of the surgical procedure, and retract the nictating membranes. Eyes were lubricated hourly with silicon ride ophthalmic solution (Akorn) was applied to both eyes to dilate the pupils 1% tropicamide ophthalmic solution (Akorn) and 10% phenylephrine hydrochloride ophthalmic solution (Akorn) and 10% phenylephrine hydrochloride ophthalmic solution (Akorn) and 10% phenylephrine hydrochloride ophthalmic solution (Akorn) and 10% phenylephrine hydrochloride ophthalmic solution (Akorn) and 10% phenylephrine hydrochloride ophthalmic solution (Akorn) and 10% phenylephrine hydrochloride ophthalmic solution (Akorn) and 10% phenylephrine hydrochloride ophthalmic solution (Akorn) and 10% phenylephrine hydrochloride ophthalmic solution (Akorn) and 10% phenylephrine hydrochloride ophthalmic solution (Akorn) and 10% phenylephrine hydrochloride. Upon completion of the surgical procedure, stainless steel retaining ring (5/16-inch internal retaining ring, McMaster-Carr), and an affine transformation. Two-photon frame triggers from Scanimage were synchronized with stimulus information using Spike2 (CED). Throughout the experiment, dendrites were carefully monitored for indications of photodamage. Individual neurons were excluded from data analysis if they exhibited nuclear fluorescence or showed prolonged calcium responses, suggesting cytotoxicity as a result of strong GCaMP expression. Typically, we acquired data from one cell per animal, except for a single experiment in which we acquired data from two cells. T-series projections were displayed as a s.d. projection of 500–2,000 frames. Images in this publication were collected from the following depths: Figure 1c – 100 μm, 500 frames, STD projection Figure 1d – 47 μm, 2,000 frames, STD projection Figure 5a, left dendrite: –57 μm, 2,000 frames, STD projection Figure 5a, middle dendrite: –154 μm, 2,000 frames, STD projection Figure 5a, right dendrite: –68 μm, 2,000 frames, STD projection Figure 6a: –80 μm, 2,000 frames, STD projection Figure 5d: –90 μm, 2,000 frames, STD projection Supplementary Figure 2a, left dendrite: –29 μm, 2,000 frames, STD projection Supplementary Figure 2a, middle and right dendrite: –28 μm, 2,000 frames, STD projection Intrinsic signal imaging. Intrinsic signal imaging was performed using a custom intrinsic signal imaging setup with a Xyla sCMOS camera (Andor) controlled by µManager2 (ref. 42). To obtain a blood vessel map, we acquired an image of the cortical surface under white light illumination. To measure intrinsic hemodynamic responses, we illuminated the surface of the cortex with a 630-nm red LED (Thorlabs). Visually driven responses were evoked using a continuously drifting and rotating square-wave grating (0.06 cycles per degree, 4 cycles s^{-1}, rotated either clockwise or counter-clockwise at 6° per second) and recorded at 50–55 Hz. Whole-cell recording. Six juvenile female ferrets (–PSO) were used for blind whole-cell patch-clamp recordings. Recordings were performed using the same cranial window setup as for two-photon imaging, but the coverage was removed and the craniomety filled with 4% agarose in ACSF. A silver/silver chloride reference electrode was inserted into the agarose and fixed to the headplate using Kwik Cast. Pipettes of 5–7 Ω resistance were pulled using borosilicate glass (King Glass) and filled with an intracellular solution containing (in mM) 135 potassium gluconate, 4 KCl, 10 HEPES, 10 disodium phosphocreatine, 4 Mg-ATP, 0.3 Na-GTP, pH 7.2, 295 mOsm. Neurons were recorded from 100 to 800 μm below the pia using a Multiclamp 700B (Molecular Devices). Series resistance and pipette capacitance were corrected online. Series resistance for recordings typically ranged from 40 to 80 MΩ. Membrane potential was digitized at 5 or 12.5 kHz using Spike2. Two-photon imaging. Images were corrected for in-plane motion using a correlation-based approach in Matlab. ROI drawing was performed in ImageJ. For somata, polygonal ROIs were manually drawn around cell bodies. For dendrites, polygonal ROIs were drawn spanning the extent of a short, contiguous dendritic segment. For spines, circular ROIs were placed over spines not overlapping with the dendritic segment. Fluorescence time courses were computed as the mean of all pixels within the ROI at each time point and were extracted using Miji (D. Sage, D. Prodanov, J. Tinevez and J. Schindelin, Miji: making interoperability between ImageJ and Matlab possible, ImageJ User & Developer Conference, 24–26 October 2012, Luxembourg, http://bigwww.epfl.ch/sage/soft/miji/). Fluorescence time courses were then synchronized with stimulus information, and visually
evoked responses were computed as changes in fluorescence relative to the last second of the ISI period.

Fluorescence signals in dendritic spines were sometimes contaminated by regenerative dendritic events. To address this, we used a computational subtraction procedure similar to one previously published20. Using only stimulus-evoked fluorescence data, we performed a robust fit (using Matlab's robustfit) of the spine fluorescence against the dendritic fluorescence and then subtracted a scaled version of the dendritic fluorescence where the scaling factor equals the slope from the robust fit. After subtraction, we enforced several inclusion criteria for spine signals: (1) mean response at the preferred orientation was >10% ΔF/F; (2) tuning was well-described by a Gaussian fit ($r > 0.7$, Pearson's correlation coefficient); (3) spine responses were weakly correlated with dendritic responses after subtraction, and (4) SNR > 1, with SNR defined43 by

$$\text{SNR} = \frac{\mu_{\text{pref}} - \mu_{\text{ortho}}}{\text{SE}_{\text{pref}} + \text{SE}_{\text{ortho}}}$$

where $\mu_{\text{pref}}$ equals the mean response to the preferred orientation, $\mu_{\text{ortho}}$ equals mean response to the orthogonal orientation, $\text{SE}_{\text{pref}}$ is the standard error of responses to the preferred orientation, and $\text{SE}_{\text{ortho}}$ is the standard error of responses to the orthogonal orientation. This SNR metric has been used previously43. Gaussian fits describing the orientation preference and orientation tuning bandwidth were obtained using Matlab's lsqcurvefit function. Orientation tuning index (OTI) was computed as

$$\text{OTI} = \frac{\mu_{\text{pref}} - \mu_{\text{ortho}}}{\mu_{\text{pref}} + \mu_{\text{ortho}}}$$

The effectiveness of the backpropagation subtraction was verified by comparing the OTI of spines with orientation preference similar to the soma ($\Delta \text{Ori}_{\text{pref}} < 15^\circ$, $n = 324$) with that of spines whose orientation preference was orthogonal to the soma ($\Delta \text{Ori}_{\text{pref}} > 75^\circ$, $n = 39$) (Wilcoxon rank-sum, $P = 0.53$). If the backpropagation subtraction was not effective, one might expect lower OTI for spines tuned orthogonally to the soma, as suggested previously20. Across all spines, OTI was not correlated with spine orientation preference relative to the soma ($r = -0.041$, $P = 0.24$, $n = 836$), further supporting the notion that the backpropagation subtraction does not generate aberrant orientation-tuned responses.

Orientation selectivity was computed as 1 – circular variance (CV) in orientation space, defined by

$$1 - \text{CV} = \frac{\sum_k \bar{R}(\theta_k) \exp (2i \theta_k)}{\sum_k \bar{R}(\theta_k)}$$

where $\theta_k$ is the orientation of a visual stimulus and $R(\theta_k)$ is the response to that stimulus.

We employed a circular dispersion metric to describe the functional clustering of dendritic spines in orientation space. To compute this, we first calculated the phase $\alpha$ of the mean resultant vector

$$\alpha = \arg \left\{ \frac{1}{K} \sum_k \exp (2i \theta_k) \right\}$$

where $\theta_k$ is the orientation preference of an individual spine and $K$ is the number of spines on a short contiguous segment of dendrite. We then calculated the branch circular dispersion44 ($D(\alpha)$) as the mean distance of spine orientation preference from this circular mean, using spines from within the same contiguous dendritic segment within a single two-photon field of view:

$$D(\alpha) = \frac{1}{2 \pi} \sum_k \left| \pi - |2 \theta_k - \alpha| \right|$$

Trial to trial correlations between spines were computed as the correlation of mean fluorescence responses to each stimulus on a per-trial basis.

To compute cellular input–output transformations, we took the arithmetic mean of dendritic spine responses of all active, orientation-tuned dendritic spines on a cell, then normalized from 0 to 1. We did the same for individual somata. We then fit orientation tuning curves to these summed responses. We set the preferred orientation for summed spine input to the somatic fluorescence data, we performed a robust fit (using Matlab's lsqcurvefit) of the spine fluorescence against the dendritic fluorescence and then subtracted a scaled version of the dendritic fluorescence where the scaling factor equals the slope from the robust fit. After subtraction, we enforced several inclusion criteria for spine signals: (1) mean response at the preferred orientation was >10% ΔF/F; (2) tuning was well-described by a Gaussian fit ($r > 0.7$, Pearson's correlation coefficient); (3) spine responses were weakly correlated with dendritic responses after subtraction, and (4) SNR > 1, with SNR defined43 by

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To identify dendritic hotspots, we drew ROIs (regions of interest) subtending the length of the dendritic shaft that was in focus within the imaging FOV (field of view) and then extracted dendritic response time courses by binning pixels at 2-μm intervals along the longitudinal axis of the dendrite. We first identified dendritic events exceeding 10% ΔF/F, regressed out the uniform baseline component of the dendritic fluorescence response, and then used a three-segment median filter to smooth the spatial responses. After removing the uniform component of the dendritic fluorescence response, we detected local dendritic events as those events whose peak exceeded five median absolute deviations above the baseline noise and measured the spatial profile of these events by fitting a Gaussian to the fluorescence response. To avoid spurious fits, we enforced several criteria: (1) the peak of the Gaussian had to be at least 6 μm from the edge of the dendritic segment, (2) the Gaussian fit had to explain at least 70% of the variance in the dendritic spatial response, and (3) the amplitude of the Gaussian needed to match the response amplitude with 5% tolerance.

**Intrinsic signal imaging.** Orientation-specific responses from 5–10 trials were extracted using a discrete Fourier transform at the stimulation frequency45:

$$z(x) = -\sum_n \left( R(x,n) - \hat{R}(x) \right) \exp \left( \frac{2 \pi i k n}{N} \right)$$

where $z(x)$ is the orientation-specific response, $N$ is the number of frames contained in a full trial (60 s), $n$ is the sequence of frame numbers ranging from 1 to $N$, $k$ is 2 (the second harmonic), $R(x,n)$ is the reflectance, and $\hat{R}(x)$ is the trial-averaged reflectance. After correction for the hemodynamic delay, orientation maps were spatially filtered with a 200-μm lowpass and 1,500-μm highpass Fermi filter in the Fourier domain:

$$\hat{z}'(x) = z(x) - F \hat{K}(k) \hat{z}(k)$$

$$\hat{K}(k) = \frac{1}{1 + \exp \left( -\left( k c_{\text{cutoff}} \right) / \beta c_{\text{cutoff}} \right) }$$

where $\hat{z}'(x)$ is the filtered orientation response, $F$ denotes the Fourier transform, $\hat{K}(k)$ is the Fermi function in Fourier space, $c_{\text{cutoff}}$ is the cutoff frequency, and $\beta$ is the steepness ($\beta = 0.05$). $z'$-projections of two-photon $z$-stacks were aligned to the blood vessel map obtained before intrinsic signal imaging using control point registration and an affine transformation. Map orientation preference ($\theta = \arg \left\{ z'(x_{\text{soma}}) \right\} ) was determined by sampling the map at the location of the soma. High gradient regions were defined as those pixels exceeding a threshold of the spatial gradient of the orientation preference map set at 11.25° per pixel, and cellular distance from high gradient regions was computed as the Euclidean distance from this region. Cells were classified as low rate of change if the soma was >100 μm from a high-gradient region and high rate of change if the soma was <100 μm from a high-gradient region.

**Whole-cell recording.** Membrane potential recordings were median filtered with a 30 to 100 sample window to remove action potentials and binned to 5 ms. $V_{m}$ and spikes were cycle-averaged and Fourier-transformed to obtain $F_{0}$ and $F_{1}$ at each stimulus, with $F_{0}$ representing the DC component of the response and $F_{1}$ representing the AC component of the response.
representing the modulation at the frequency of the stimulus. $V_m$ and spiking responses were computed relative to mean spontaneous responses. To measure the distance from resting membrane potential to spike threshold, we took the response to the blank as the resting membrane potential, and measured the location of the ‘kink’ in the AP waveform for 10 spikes per cell.

**Statistics.** Statistical analyses were performed in Matlab. We used two-sided non-parametric Wilcoxon rank-sum tests or two-sample Kolmogorov-Smirnov tests to compare two groups. Correlation coefficients were calculated as Pearson’s unless otherwise specified; exact sample sizes are included in the text. No estimates of statistical power were performed before experiments; animal numbers were minimized to conform to ethical guidelines while accurately measuring parameters of animal physiology. No randomization was used in analysis. Intrinsic signal imaging was performed after two-photon imaging, so the experimenter was blind to cellular location in the orientation preference map during data acquisition.

**Data availability.** The data that support the findings of this study are available from the corresponding author upon request.

**Code availability.** Analysis code was written using standard Matlab functions and MIJ. Code is available upon request.