Anatomy and function of an excitatory network in the visual cortex

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Circuits in the cerebral cortex consist of thousands of neurons connected by millions of synapses. A precise understanding of these local networks requires relating circuit activity with the underlying network structure. For pyramidal cells in superficial mouse visual cortex (V1), a consensus is emerging that neurons with similar visual response properties excite each other¹⁻⁴, but the anatomical basis of this recurrent synaptic network is unknown. Here we combined physiological imaging and large-scale electron microscopy to study an excitatory network in V1. We found that layer 2/3 neurons organized into subnetworks defined by anatomical connectivity, with more connections within than between groups. More specifically, we found that pyramidal neurons with similar orientation selectivity preferentially formed synapses with each other, despite the fact that axons and dendrites of all orientation selectivities pass near (<5 μm) each other with roughly equal probability. Therefore, we predict that mechanisms of functionally specific connectivity take place at the length scale of spines. Neurons with similar orientation tuning formed larger synapses, potentially enhancing the net effect of synaptic specificity. With the ability to study thousands of connections in a single circuit, functional connectomics is proving a powerful method to uncover the organizational logic of cortical networks.

Pyramidal cells in the rodent primary visual cortex (V1) respond to highly specific visual features, resulting in diverse receptive field preferences, in contrast with the less selective responses of most inhibitory neurons⁵⁻¹⁰. A model is emerging in which V1 responses arise from the selective amplification of thalamocortical signals¹¹⁻¹⁴ through recurrent inputs from other pyramidal neurons²,⁵,¹⁴,¹⁵. Evidence for functionally specific cortical amplification has been seen in physiological²,³,¹⁴ and optogenetic⁴ studies; however, firm anatomical evidence at the synaptic level has been lacking (but see ref. 5). A recent study also showed that neurons with similar orientation preference share stronger connections¹⁵; however, it is unknown if this effect is due to stronger synapses, more synapses, or perhaps spatially clustered synapses.

To test these hypotheses, we measured the receptive-field properties and reconstructed the detailed anatomy of the same visual cortical neurons, identifying actual synapses versus axonal-dendritic appositions⁶ (Fig. 1). We combined in vivo cellular resolution optical imaging with ex vivo electron microscopy (EM) reconstructions. We measured cellular calcium responses, which reflect the firing of action potentials, using the genetically encoded indicator GCaMP3 to characterize the sensory responses of ~300 μm × 300 μm × 200 μm volume of an awake, behaving mouse¹⁶ (Fig. 1b–c). Visual stimuli consisted of drifting sinusoidal gratings of different spatial and temporal frequencies, orientations, and directions (Fig. 1b and Extended Data Fig. 1). In addition to cell bodies in layers 2/3 (L2/3), we measured signals (Extended Data Fig. 2) from large calibre apical dendrites that continued beyond the depth of our imaging volume and had branching morphologies consistent with deep layer pyramidal cells (see Methods). From their responses, we estimated the peak preferred orientation for each cell (Extended Data Fig. 3), with neurons’ visual preferences typically maintained across 12 days of chronic imaging (Extended Data Fig. 1).

After locating the functionally imaged region using vascular landmarks (Extended Data Fig. 4 and Supplementary Video 1), we cut a series of ~3,700 serial EM sections, which were imaged with a transmission electron microscope camera array⁸ (TEMACA) at ~4 nm × 4 nm × 40 nm per voxel. The EM-imaged region spanned 450 μm × 450 μm × 150 μm, consisting of ~10 million camera images and ~100 TB of raw data. We traced the processes of excitatory pyramidal neurons located within the middle third of the EM volume (450 μm × 450 μm × 50 μm, Supplementary Videos 2 and 3) using software (CATMAID¹⁷) allowing distributed annotation of large image data sets. Teams of trained annotators traced and validated wire-frame models of the dendritic and axonal arbors of neurons selected for reconstruction (chosen because they exhibited visual responses), and located all outgoing synapses along the annotated axons (Fig. 1d, and Extended Data Figs 5 and 6a). For each synapse, we traced the postsynaptic dendrite centripetally until they reached either the cell body or the boundary of the aligned EM volume (Extended Data Fig. 6b, and Supplementary Data 1–3). We did not retrogradely trace axons providing input to selected neurons because of the lower probability that they originated from cells in the volume. Henceforth, we limit our discussion to the excitatory network from selected pyramidal cells onto spines of excitatory targets.

We first examined the structure of the network broadly, by examining the connectivity of 1,278 reconstructed neuronal targets (Table 1). The core of the network (201 neurons connected with more than one other; that is, degree ≥ 2, no leaf nodes) exhibited a modular structure (Fig. 1e), quantified with a modularity index (Q), which can range from −0.5 to 1 that measures the degree to which a network can be divided into groups, with positive values indicating higher levels of connectivity within than between groups¹⁷. Modularity in the reconstructed network was significantly higher than would be expected by chance (Qmean = 0.55 ± 0.003 vs 0.50 ± 0.009, mean ± s.d., P≈ 0, permutation test; Fig. 1e and Extended Data Fig. 7a–d, see Methods) and was not a consequence of higher order motifs (Extended Data Fig. 7). Modularity of the network offers positive evidence for pyramidal cell subnetworks that have previously been inferred from physiological recordings²,¹⁸,¹⁹.

We next examined the degree to which network connectivity reflects the neurons’ sensory properties (Figs 1f and 2). We focused on 46 L2/3 excitatory neurons and 4 deep layer apical dendrites that showed reproducible responses to visual stimulation (trial correlations for spatial and temporal frequency or stimulus position experiments, Pdiff < 0.05 and Ppos < 0.01, respectively, excluding cells with stimulus edge effects, see Methods) and identified their postsynaptic targets. Within the reconstructed network, 43 functionally characterized neurons made 990 synapses, 443 of which were onto inhibitory interneurons and 547 onto other pyramidal cells (Table 1). The large fraction of synapses onto

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**Figure 1 | Functional organization of cortical excitatory network connectivity.** a, Schematic representation of functionally selective connections between excitatory neurons. Excitatory pyramidal cells (large circles) with different preferred orientations provide synaptic input (smaller circles) to one another (bottom, colour code used throughout to indicate stimulus orientation that evokes peak physiological responses). b, Example stimuli (top) and time courses of $\Delta F/F$ signals from single neurons. c, Combined in vivo two-photon calcium imaging and electron microscopy (EM). Top left, schematic of imaging in the awake mouse, targeting the monocular region of the primary visual cortex (largest dotted-outlined region surrounded by higher visual areas). Red arrows represent the visual pathway from eye to visual thalamus to cortex. Serial EM sections (right) were cut orthogonal to the functional imaging planes (left). d, Left, reconstruction of a layer 2/3 (L2/3) pyramidal neuron (cell 1) synapsing onto a deep layer apical dendrite (cell 2), reconstructed from EM (colour denotes preferred orientation of neuron). The thinnest process is the axon, dendrites of the L2/3 neuron are thicker, and the deep layer apical dendrite is rendered with the largest calibre. Dendritic spines were traced only if they participated in connections between reconstructed neurons. Synapses are shown as small spheres. Presynaptic inhibitory neurons is consistent with past observations\(^5,20\) and seems to be a characteristic of L2/3 in the mouse visual cortex. Consistent with physiological studies, connectivity between excitatory neurons was more likely for pairs with similar orientation preferences\(^2\) (Fig. 2b, $P < 0.05$, Cochran–Armitage test). This preferential connectivity is not explained by the anatomical arrangement of the cell bodies whose function showed no discernible dependence on distance (Extended Data Fig. 8).

As axons and dendrites of physiologically characterized neurons were traced exhaustively within the volume, we could further evaluate in an unbiased fashion a potential role of axonal and dendritic geometry in specific connectivity. We used a modified measure of potential synapses\(^21\), which our reconstructions allowed us to compute directly. For each pair of neurons, we quantified potential synaptic length, or the length that each dendrite ($L_d$) travelled within a distance ($s$) from a given axon, where $s$ can be considered a maximum distance a spine could reach to make a connection ($s = 5\,\mu m$: Fig. 2a, c; $s = 1\,\mu m$: Extended Data Fig. 9; see Methods). This helps us formulate a fine-scale version of Peters’ rule: the hypothesis that the probability of a connection is proportional to the degree of axo-dendritic proximity, independent of other factors. As expected, connected neurons had significantly more $L_d$ than unconnected pairs, that is, their geometry provided more opportunities to make synapses ($P \approx 0$, permutation tests, $s = 5\,\mu m$, Fig. 2c; $s = 1\,\mu m$: Extended Data Fig. 9). More interestingly, by considering potential synaptic length between pairs with different relative orientations, we found that the neuropil is not functionally

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organized (P > 0.5, permutation tests relative to a uniform distribution, s = 5 μm: Fig. 2d, black line, and 2e; s = 1 μm: Extended Data Fig. 9). Therefore, the preferential connectivity (Fig. 2d, red versus blue line and red versus black line both, P < 0.05, permutation tests; and Fig. 2f, P < 0.05, Cochran–Armitage test) between neurons with similar orientation preferences must be the result of mechanisms that take place at the scale of spines: ~1 to 5 μm. Because synapses onto the apical dendrites of deep layer neurons might follow different rules, we also confirmed statistical significance (P < 0.05, permutation test, data not shown) with only L2/3 connections.

Recent work suggests that, along with connection probability, the amplitude of excitatory postsynaptic potentials (EPSPs) correlates with receptive field properties. Mechanisms underlying the strength of these unitary responses are unknown, for instance whether they are due to multiple synapses, the spatial organization of their synapses, or stronger synapses. Although our sample size was not sufficient to examine the relationship between multiple synapses and function, past work demonstrated that virtually all pyramidal cells are connected by multiple synapses (reviewed in ref. 23). We therefore examined whether synapses connecting pairs of neurons were spatially clustered (Fig. 3a, Fig. 1d, f, thick arrows, and Supplementary Data 3) in a population of 51 reconstructed presynaptic neurons connected by multiple synapses onto one or more postsynaptic targets (Table 1). We first computed the synapse rate (λ), or synapses per 100 μm of L4. As connected, connected neurons coupled by multiple synapses occur more frequently than predicted by chance, having far higher synapse rates for additional synapses compared to a Poisson model (P ≈ 0, permutation tests relative to a Poisson process with λ, Fig. 3b, compared to Fig. 2f, see Methods). Next, we found that pyramidal neurons were frequently interconnected by multiple synapses arranged close together (17.2 ± 2.9 μm, median ± s.e.m., Fig. 3c) consistent with recent analyses of axon fragments in L5 apical dendrites and hippocampal neurons. A careful analysis of synapse locations considering potential synapse length (L5), however, shows that closely spaced synapses are not specifically enriched. By comparing the relative magnitude of L5 for regions

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**Table 1 | Cortical network reconstruction**

| Functionally characterized neurons | 50            |
|-----------------------------------|---------------|
| Characterized connected neurons   | 43            |
| Characterized deep layer apicals  | 4             |
| Characterized neurons connected to deep layer apicals | 20 |
| Functionally characterized connected pairs | 29 |
| Unique characterized presynaptics | 15            |
| Unique characterized postsynaptics | 21            |
| Characterized pairs containing deep layer apicals | 8 |
| Unique characterized deep layer apicals in pairs | 2 |
| Synapses from characterized neurons | 990          |
| Synapses onto inhibitory targets  | 443           |
| Synapses onto excitatory targets  | 547           |
| Reconstructed PSDs between characterized pairs | 39 |
| Multi-synapse connected pairs     | 115           |
| Multi-synapse connected pairs with deep layer apicals | 62 |
| Multi-synapse connected presynaptic neurons | 51 |
| Multi-synapse connected postsynaptic non-deep layer apicals | 52 |
| Multi-synapse connected postsynaptic deep layer apicals | 41 |

Numbers of reconstructed neuronal targets (neurons and dendritic fragments), synaptically connected neuron pairs, and synapses analysed in the EM data set.

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**Figure 2 | Synaptic connectivity between pyramidal cells predicted by function, over and above proximity. a,** Reconstructions of three neurons with regions of potential synaptic connectivity (L5 dotted outlines), which quantify the dendritic path length of cells 2 and 3 within a maximal spine length (s = 5 μm) of the axon of cell 1. Neurons colour-coded by preferred stimulus orientation (colour key, bottom left; same as Fig. 1). Note, the large L4, but lack of actual synapses between cells 1 and 2 and small L4 between cells 1 and 3 where an actual synapse (arrow) is observed along the L4. The axon of the postsynaptic cells and dendrites of the presynaptic cell are transparent. Only presynaptic boutons (small red spheres with white centres) from the presynaptic cell are visible. **b,** Synaptically connected neurons have similar orientation preference. Connection probability as function orientation preference difference across the reconstructed population (P < 0.05, Cochran–Armitage test, nconnected pairs = 29, nunconnected pairs = 1,951). Bins include pairs of neurons with differences in preferred orientation of 0° to 22.5°, 22.5° to 45°, 45° to 67.5°, and 67.5° to 90°. Fractions at the bottom of bars are the number of connected over unconnected pairs in each bin. **c,** Synaptically connected neurons have greater potential synapse length. Cumulative distribution of potential synapse length was significantly greater between connected (red line) than unconnected pairs (blue line, P ≈ 0, permutation test, nconnected pairs = 29, nunconnected pairs = 1,951, s = 5 μm). Inset, schematic of a cylinder of length L4 (transparent purple) around the dendrite (red) with a radius (s) equivalent to a maximal spine’s length where the axon (blue) comes within proximity to make a synapse. **d,** Cumulative distribution of differences in orientation preference was significantly less between connected (red line) than unconnected pairs (blue line, P < 0.05, permutation test, nconnected pairs = 29, nunconnected pairs = 1,951) or a model distribution based on potential synapse length (black line, P < 0.05, permutation test, nconnected pairs = 29, nunconnected pairs = 1,951). **e,** Potential synapse length (L4) is uniform across differences in peak orientation preference between neurons in the model distribution. **f,** Synapse rate (λ) reconstructed synapses normalized by L4 decreases with orientation preference difference across the reconstructed population (P < 0.05, Cochran–Armitage test, nconnected pairs = 39, s = 5 μm). Error bars in b, f, shaded regions in c, d represent bootstrapped standard error of the mean. Scale bar, a, 100 μm.
>20μm versus <20μm from each synapse between a pair of neurons, we found that the number of distant synapses correlated ($P \approx 0$, permutation test, $n_{\text{synapses}} = 195$, Pearson’s $r = 0.92$) nearly perfectly with the expected value from the pair’s average density of synapses (Fig. 3d, see Methods). That is, axons and dendrites of connected neurons generate multiple synapses when their axon and dendrite remain close, but make additional synapses with an equal rate ($\lambda$) elsewhere their processes overlap within our limited volume. This suggests plasticity mechanisms that operate at the cellular rather than dendritic level, but nonetheless leaves open a potential computational role for positioning mechanisms that operate at the cellular rather than dendritic level, but nonetheless leaves open a potential computational role for positioning multiple synapses, which occur both clustered and distributed along the dendritic arbor.

Finally, we examined synapse size by measuring postsynaptic density (PSD) area (Fig. 4a), which is proportional to the number of presynaptic vesicles and spine volume24, and may be related to synaptic strength27-29. We found that PSD areas could be large (>0.25μm$^2$) for synapses between cells with similar peak orientations (Fig. 4b, c), but were clustered at a smaller size (0.06 ± 0.008μm$^2$, mean ± s.e.m.) for the most dissimilar orientations. Connected cells (Fig. 4c, red line) with similar peak orientation preference exhibited larger PSD areas compared to control populations where the orientation selectivity differences or identity of connected cells were shuffled (Fig. 4c, cyan and magenta lines, respectively, $P < 0.01$, permutation tests). This functionally specific synaptic weighting was significant whether or not spines from apical dendrites were included in the analysis (data not shown). One possibility is that there are multiple classes of synapses, latent connections with smaller synapses, and larger synapses between similarly tuned cells.

Functional connectomics promises to build bridges between the in vivo activity of neurons, network connectivity, and neuronal
structure. Here, we used this approach to demonstrate five aspects of the excitatory cortical circuit. Independent of physiology, we demonstrate (1) a modular network organization that previously had only been inferred indirectly from smaller cortical subnetworks, and (2) that multiple synapses between pairs of neurons occur far above chance levels, often closely spaced on the dendrite, although there seems to be no specific mechanism favouring local clustering over widespread spacing. Further, we demonstrate (3) an anatomical substrate of functionally specific connections between neurons, and (4) that this specificity does not result from the spatial arrangement of the neuropil, but instead must operate at the scale of dendritic spines. Finally, we show (5) that synapse size correlates with physiology, with larger synapses found between neurons with similar peak orientations. Such specific connectivity is consistent with intracortical amplification of afferent signals, overcoming the strong inhibitory tone in the awake cortex. As methods for automated reconstruction improve, each of these findings will come into greater focus, leading to a richer understanding of network structure and function.

Online Content Methods, along with any additional Extended Data display items and Source Data, are available in the online version of the paper; references unique to these sections appear only in the online paper.

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Author Contributions W.-C.A.L. and V.B. performed the in vivo calcium imaging and analysed it. W.-C.A.L. processed the tissue for EM, sectioned the series, and aligned the block with the in vivo imaging. W.-C.A.L. and M.R. imaged it on the TEMCA-G.H. and W.-C.A.L. aligned the EM images into a volume, W.-C.A.L., M.R., K.G. annotated the EM dataset and W.-C.A.L. and K.G. supervised segmentation efforts. B.J.G. and W.-C.A.L. generated software for visualization and analysis. W.-C.A.L. performed quantitative analysis on the tracing. W.-C.A.L., VB., and R.C.R. designed the experiment and wrote the paper.

Author Information The aligned EM dataset will be publicly accessible at http://neurodata.io/lee16. Reprints and permissions information is available at http://www.nature.com/reprints. The authors declare no competing financial interests. Readers are welcome to comment on the online version of the paper. Correspondence and requests for materials should be addressed to W.-C.A.L. (wei-chung.lee@hms.harvard.edu) or R.C.R. (clayr@alleninstitute.org).
METHODS

We imaged the primary visual cortex of an awake 9-month-old C57BL/6 male mouse, as described previously, with a custom-built two-photon microscope. Using volumetric in vivo two-photon calcium imaging of a genetically encoded calcium indicator (GCaMP3), we measured the time-resolved responses of a population of identified neurons to a wide array of stimuli including drifting gratings (up to 16 directions, 3 spatial, and 2 temporal frequencies). Following 12 days of imaging calcium responses in the same cohort of neurons, we labeled blood vessels using intravital injection (rhodamine B-conjugated dextran) and acquired an in vivo fluorescence volume. The animal’s brain was then prepared for large-scale transmission EM as described previously. During imaging sessions (~<50 nm thick) were cut and imaged spanning a 450 μm × 450 μm × 150 μm volume at 4 nm × 4 nm × 40 nm per voxel resolution. Sections representing the middle third of the EM volume were aligned and imported into CATMAID for distributed, online, manual reconstruction and targeted volumes around identified synapses were exported for volumetric segmentation and PSD analysis. EM reconstructed neurons were identified in the in vivo stack by using the blood vessels as landmarks. Apical dendrites originating from deeper neocortical lamina were similarly identified and corresponded by location and branching geometry of their apical tufts. Permutation tests were used in statistical analyses, unless otherwise noted.

Animal preparation. All procedures were conducted in accordance with the ethical guidelines of the NIH and approved by the IACUC at Harvard Medical School. For cranial window implant surgery the mouse was anesthetized with isoflurane (1–1.5%). We performed retinotopic mapping of visual cortical areas using widefield intrinsic signal imaging. Using volumetric calcium signals in vivo, we identified L4 and L5 pyramidal cells. These were likely from L5 neurons because of their large calibre, and because most L6 pyramidal cells do not project their apical dendrites more superficially than L4. The calcium signals from these deep layer apical dendrites stem from either forward- or back-propagating action potentials, are consistent across days and along the length of the deep layer pyramidal cells. These were likely from L5 neurons because of their large calibre, and because most L6 pyramidal cells do not project their apical dendrites more superficially than L4. The calcium signals from these deep layer apical dendrites stem from either forward- or back-propagating action potentials, are consistent across days and along the length of the deep layer pyramidal cells. These were likely from L5 neurons because of their large calibre, and because most L6 pyramidal cells do not project their apical dendrites more superficially than L4. The calcium signals from these deep layer apical dendrites stem from either forward- or back-propagating action potentials, are consistent across days and along the length of the deep layer pyramidal cells. These were likely from L5 neurons because of their large calibre, and because most L6 pyramidal cells do not project their apical dendrites more superficially than L4. The calcium signals from these deep layer apical dendrites stem from either forward- or back-propagating action potentials, are consistent across days and along the length of the deep layer pyramidal cells. These were likely from L5 neurons because of their large calibre, and because most L6 pyramidal cells do not project their apical dendrites more superficially than L4. The calcium signals from these deep layer apical dendrites stem from either forward- or back-propagating action potentials, are consistent across days and along the length of the deep layer pyramidal cells. These were likely from L5 neurons because of their large calibre, and because most L6 pyramidal cells do not project their apical dendrites more superficially than L4. The calcium signals from these deep layer apical dendrites stem from either forward- or back-propagating action potentials, are consistent across days and along the length of the deep layer pyramidal cells. These were likely from L5 neurons because of their large calibre, and because most L6 pyramidal cells do not project their apical dendrites more superficially than L4. The calcium signals from these deep layer apical dendrites stem from either forward- or back-propagating action potentials, are consistent across days and along the length of the deep layer pyramidal cells. These were likely from L5 neurons because of their large calibre, and because most L6 pyramidal cells do not project their apical dendrites more superficially than L4. The calcium signals from these deep layer apical dendrites stem from either forward- or back-propagating action potentials, are consistent across days and along the length of the deep layer pyramidal cells. These were likely from L5 neurons because of their large calibre, and because most L6 pyramidal cells do not project their apical dendrites more superficially than L4.

EM material preparation. Following in vivo two-photon imaging the animal was perfused transcardially with 0.04% CaCl2) and the brain was processed for serial-section TEM. The animal’s brain was then prepared for large-scale TEM imaging, alignment, and correspondence. Using the custom-built transmission electron microscope camera array (TEMA), we imaged the ~3,700 serial section series, targeting a ~450 μm × 540 μm region for each section (Fig. 1c). Acquired at 4 nm per pixel in plane, this amounted to ~100 terabytes of raw data to date comprising 30 million cubic microns of brain and ~10 million (4,000 × 2,672 pixel) camera images. Magnification at the scope was 2,000 ×, accelerating potential was
120 kV, and beam current was ~90 microamperes through a tungsten filament. Images suitable for circuit reconstruction were acquired at a net rate of 5–8 million pixels s$^{-1}$. Approximately the middle third of the series (sections 2,281–3,154) was aligned using open source software developed at Pittsburgh Supercomputing Center (AlignTK) and imported into CATMAID$^{46}$ for distributed online visualization and segmentation. Within the analysed EM series there were 51 missing sections. Nineteen were single section losses. There were 2 instances each of missing 2, 3, and 4; and 1 instance each of missing 6 or 8 consecutive sections near the series boundaries. The staining approach was slightly different, superimposed during section processing, but were typically isolated to edges of our large sections and therefore did not usually interfere with manual segmentation. To find the correspondence between the cells imaged in vivo with those in the EM data set, a global 3D affine alignment was used with fiducial landmarks manually specified at successively finer scales of vasculature and then cell bodies to re-locate the calcium–imaged neurons in the EM-imaged volume (Extended Data Fig. 4). Apical dendrites arising from deep layer (putative L5) pyramidal neurons were identified by their characteristic morphology$^{34,43}$ (also see below). Their correspondence was facilitated by the unique branching patterns of their apical tufts and those that could not be unambiguously identified were not included in the functional analysis.

**Reconstruction and verification.** We first traced the axonal and dendritic arbors of the functionally characterized neurons in the EM data set by manually placing a series of markers points down the midline of each process to generate a skeletonized model of the arbor using CATMAID$^{46}$ (Figs 1d, 2a, 3a, Extended Data Fig. 6, Supplementary Data 1–3). We identified synapses using classical criteria$^{42}$. For each synapse on the axon of a functionally characterized cell, dendrites of postsynaptic excitatory neurons were traced either to the boundaries of the aligned volume or centripetally back to the cell body$^{9}$. We identified deep layer apical dendrites of (putative L5) pyramidal cells by their large calibre, high spine density, and their continuous dendritic arbor beyond the bottom border of the EM volume, which spread from the pial surface through layer I4. For each neuronal target reconstruction included in the analysis, a second independent annotator verified the tracing by working backwards from the most distal end of every process. An additional round of validation was done for each synapse between functionally characterized cells where a third annotator who had not previously traced the pre- or post-synaptic process, independently verified the anatomical connectivity blind to previous tracing work. We began this independent round of validation at each synapse and traced the pre- and post-synaptic processes centripetally. If the initial reconstruction and subsequent verification of the reconstruction diverged, that connection and the segmentation work distal from the point of divergence was excluded from further analysis. EM reconstruction and validation was performed blind to cells’ functional characteristics and targeted cells were initially assigned to individual annotators pseudo-randomly weighted by tracing productivity.

We performed targeted volumetric reconstructions of synapses connecting functionally characterized cells by developing tools to interface with CATMAID cutout, locally align, and catalogue volumes of interest based on location (Fig. 4a; for example, 400 pixels \times 400 pixels \times 41 sections or 3.2 \mu m \times 3.2 \mu m \times 1.64 \mu m volumes centred on synapses represented by CATMAID connectors). Presynaptic boutons, postsynaptic spines, their parent axons and dendrites, and postsynaptic density (PSD) areas were manually segmented with ITK-SNAP (http://www.itksnap.org/). PSD areas were calculated as described previously$^{43}$ with obliquely cut or en face synapse areas measured using their maximum z-projection. En face or obliquely cut synapses were identified by serial sections that starkly transitioned from a clear presynaptic specialization hosting a vesicle pool, to a distinctly different post-synaptic cell, typically with an intervening section of electron dense area representing the postsynaptic density and/or synaptic cleft (for example, Extended Data Fig. 5).

**Data analysis.**

*In vivo* calcium imaging data was analysed in MATLAB and ImageJ (NIH) and modified from Brain Connectivity Toolbox$^{44}$.

To correct for motion artefact (x–y motion), the stack for each imaging plane was registered to the average field of view using TurboReg$^{44}$. A 5 pixel border at each edge of the field of view was included using TurboReg 44 . A 5 pixel border at each edge of the field of view was included using TurboReg 44 . A 5 pixel border at each edge of the field of view was included using TurboReg 44 . A 5 pixel border at each edge of the field of view was included using TurboReg 44 . A 5 pixel border at each edge of the field of view was included using TurboReg 44 .

Potential synapse length ($L_d$) represents the degree to which pairs of neurons’ axonal and dendritic arbor comes sufficiently close to make a synapse (Fig. 2a, c, 9b, d). Extended Data Figs 9, 10). For excitable pyramidal cells, we computed this length of potential synaptic connectivity between all pairs by first resampling the dendritic and axonal arbor skeletons to a maximum segment length of 40 nm (the average thickness of the EM sections) and summing the length of all dendrite segments within a maximum spine length distance of the axon ($s = 5 \mu m$: Figs 2, 3 and Extended Data Fig. 10; $s = 1 \mu m$: Extended Data Fig. 9). We use $s = 5 \mu m$ based the longest spine connecting functionally connected neurons ($\sim 5 \mu m$).

Analysis of neurons connected by multiple synapses (Fig. 3) was not restricted to cell pairs where both pre and post-synaptic cells were physiologically characterized. This population included 137 neurons connected by 267 synapses in 115 multi-synapse cell pairs whose axonal and dendritic arbor were traced exhaustively in the aligned volume. As a comparison population, we used 29 connected pairs. To examine whether poly-synaptic connectivity occurs greater than random, we first computed a population average synapse rate ($\lambda_{av}$) normalized by potential synapse...
length, by dividing the total number of synapses reconstructed from the set of 50 functionally characterized neurons by their total pairwise $L_d$. We next compared $\lambda$ for individual neuron pairs each connected by different numbers of synapses (Fig. 3b). This was used to assess whether multiple synapses occurred more often than predicted from a simple Poisson model.

We examined the frequency of clustered vs distant synapses by comparing synapse pairs that were separated by $>20\mu$m or $<20\mu$m. For each synapse from each pair of neurons connected by $n$ synapses, we computed the total $L_d$ within $20\mu$m or beyond $20\mu$m from that synapse. We then took the fraction of the overlap beyond $20\mu$m:

$$\frac{L_d (>20\mu m)}{L_d (<20\mu m) + L_d (>20\mu m)}$$

as the expected probability that each of the $(n-1)$ other synapses will occur $>20\mu$m away. The expected number of distant synapse was taken as $(n-1)$ times the fraction of overlap beyond $20\mu$m, which was compared with the actual number of distant synapses observed (Fig. 3d).

3D renderings were generated using Blender (http://www.blender.org/) (Figs 1d, 2a, 3a, Extended Data Fig. 6, Supplementary Data 1–3), Imaris (Bitplane) (Extended Data Fig. 4 and Supplementary Video 1), and ITK-SNAP (Fig. 4a). Cytoscape (http://www.cytoscape.org/) was used for network graph layouts (Figs 1f).

Statistics. Statistical methods were not used to predetermine sample sizes. Statistical comparisons between sample distributions were done with Permutation tests (that is, Monte Carlo-based Randomization tests) unless otherwise noted. Permutation tests were ideal as we do not assume the underlying distributions are normal, nor need the observations to be independent. For Permutation tests, we computed the incidence of differences between means or Pearson’s linear correlation coefficient of randomly drawn samples from combined sample distributions exceeding the empirical difference (Figs 2b–d, f, 4c, and Extended Data Figs 7b, 9a, b, 10c, d). Cochran-Armitage two-sided tests for trend were used on proportional binned data with linear weights (Fig. 2b, f). Standard errors were calculated from bootstrapped sample distributions. For cumulative distributions (Figs 2c, d, 4c, and Extended Data Figs 9a, b, 10c, d), we repeatedly resampled by randomly drawing with replacement from the sample distribution the number of observed values $1,000–10,000$ times and extracted the standard deviation at each step of the empirical CDF. For binned data (Fig. 2b, f, and Extended Data Fig. 3d), each resampled distribution was binned and the standard deviation was computed from the resampled probabilities or rates within each bin.

Code availability. Custom code is available upon request.

32. Andermann, M. L., Kerlin, A. M. & Reid, R. C. Chronic cellular imaging of mouse visual cortex during operant behavior and passive viewing. Front. Cell. Neurosci. 4, 3 (2010).

33. Tian, L. et al. Imaging neural activity in worms, flies and mice with improved GCaMP calcium indicators. Nature Methods 6, 875–881 (2009).

34. Mank, M. et al. A genetically encoded calcium indicator for chronic in vivo two-photon imaging. Nature Methods 5, 805–811 (2008).

35. Andermann, M. L. et al. Chronic cellular imaging of entire cortical columns in awake mice using microprisms. Neuron 80, 900–913 (2013).

36. Peters, A. & Kara, D. A. The neuronal composition of area 17 of rat visual cortex. I. The pyramidal cells. J. Comp. Neurol. 234, 218–241 (1985).

37. Ferrer, I., Fabregues, I. & Condor, E. A Golgi study of the sixth layer of the cerebral cortex. I. The lissencephalic brain of Rodentia, Lagomorpha, Insectivora and Chiroptera. J. Anat. 145, 217–234 (1986).

38. Hirsch, J. A., Alonso, J. M. & Reid, R. C. Visually evoked calcium action potentials in cat striate cortex. Nature 378, 612–616 (1995).

39. Smith, S. L., Smith, I. T., Branco, T. & Hausser, M. Dendritic spikes enhance stimulus selectivity in cortical neurons in vivo. Nature 503, 115–120 (2013).

40. Markram, H., Heim, P. J. & Sakmann, B. Dendritic calcium transients evoked by single back-propagating action potentials in rat neocortical pyramidal neurons. J. Physiol. (Lond.) 485, 1–20 (1995).

41. Larkman, A. & Mason, A. Correlations between morphology and electrophysiology of pyramidal neurons in slices of rat visual cortex. Establishment of cell classes. J. Neurosci. 10, 1407–1414 (1990).

42. Peters, A., Palay, S. L. & Webster, H. d. The fine structure of the nervous system: neurons and their supporting cells 3rd edn (Oxford Univ. Press, 1991).

43. Harris, K. M. & Stevens, J. K. Dendritic spines of the cerebellar Purkinje cells: serial electron microscopy with reference to their biophysical characteristics. J. Neurosci. 8, 4455–4469 (1988).

44. Thévenaz, P., Ruttimann, U. E. & Unser, M. A pyramid approach to subpixel registration based on intensity. IEEE Trans. Image Process. 7, 27–41 (1998).

45. Rubinov, M. & Sporns, O. Complex network measures of brain connectivity: uses and interpretations. NeuroImage 52, 1059–1069 (2010).

46. Lancichinetti, A. & Fortunato, S. Consensus clustering in complex networks. Sci. Rep. 2, 336 (2012).

47. Maslov, S. & Sneppen, K. Specificity and stability in topology of protein networks. Science 296, 910–913 (2002).

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Extended Data Figure 1 | Sensory physiology is maintained over days.

**a**, Visually evoked calcium responses are maintained across days. Three example neurons (rows) on the first, ninth, and twelfth day (columns) of in vivo two-photon calcium imaging. Three individual trial responses (black lines) of 8 directions, 3 spatial and 2 temporal frequencies (left column, day 1); or 16 directions, 2 spatial and 2 temporal frequencies (middle and right rows, days 9 and 12). Scale bars, 100% \( \Delta F/F \) and 4 s. Standard deviation of preferred direction across days for each neuron is to the upper right of activity matrices. **b**, Neurons direction selectivity is stable over days. Cumulative distribution of standard deviation of peak preferred direction (4.1° ± 1.7°, median ± s.e.m.) across days for 25 neurons measured over multiple days.
Deep layer apical responses are likely suprathreshold reflecting activity at the soma. Example $\Delta F/F$ time courses from a deep layer apical dendrite optically sectioned in vivo across 6–8 planes. Note, activity is correlated across depth and relatively stable over days (Extended Data Fig. 1).
Extended Data Figure 3 | Distribution of orientation and direction tuned cells. a, b, Histograms of functionally characterized cells with measured peak orientation (a) and direction preference (b).
Extended Data Figure 4 | *In vivo* to EM correspondence of neuronal targets. Top row, volumetric projections of the aligned *in vivo* and EM imaged volumes. Physiology planes were acquired horizontally and EM sections cut frontally (coronally) from the brain. Interdigitated physiology planes are from 2 representative volumetrically scanned experiments stacked atop one another in space (Fig. 1c) so as to span from the border of L1 and L2 through the depth of L2/3. Scale bar, 100 μm. Middle and bottom rows, re-sliced planes through the *in vivo* volume corresponding to EM sections. Arrowheads indicate matching cell bodies, and arrows deep layer (putative L5) apical dendrites. Small black dots mark the centres of cell bodies corresponding principally to nuclei where calcium indicator fluorescence is typically excluded. Scale bar, 50 μm.
Extended Data Figure 5 | En face synapse example. Serial sections from an obliquely cut synapse (Fig. 1d, top right, same scale). Colour overlays correspond to peak orientation preference (colour key, same as Fig. 1a, bottom).
Extended Data Figure 6 | Network reconstruction. 3D rendering of dendrites and axons, cell bodies (large spheres), and synapses (small spheres) of a, 50 functionally characterized neurons reconstructed in the EM volume. Cell bodies, dendrites, axons, and synapses colour coded by peak preferred stimulus orientation (colour key, bottom right). Axons are the thinnest processes, dendrites of L2/3 neurons are thicker, and deep layer apical dendrites are rendered with the largest calibre. Dendritic spines were traced only if they participated in connections between reconstructed neurons. b, Approximately 1,800 additional neuronal targets reconstructed in the EM volume (transparent grey). Input and output synapses are coloured cyan and red respectively when orientation selectivity was not known. Bounding box matches region in Fig. 1f. Scale bar, 150 μm.
Extended Data Figure 7 | Network modularity is significantly non-random. a, Connectivity matrix of 201 excitatory neuronal targets in our network reconstruction with multiple synaptic partners (that is, degree ≥ 2, no leaf nodes, same as Fig. 1e). Colour represents the number of synapses (colour key, c, right) between pre- and post-synaptic neurons (same neuron order on both ordinate and abscissa). Subnetworks of interconnected neurons (white boxes) detected using a consensus method of Louvain clustering17,31. b, Modularity (Q) of the reconstructed network is significantly greater than null models with degree, weight, and strength preserved. Histograms of the modularity values for the reconstructed network (dark grey, Qmean = 0.55 ± 0.003, mean ± s.d., computed 1,000 times) is significantly greater than for the Qmean of shuffled null models (light grey, Qmean = 0.50 ± 0.009, mean ± s.d., P ≈ 0, permutation test, kshuffles = 1,000). c, Example of the shuffled connectivity matrix with a Q closest to the mean of the shuffled distribution with clustering (white boxes) computed as in a. d, Null models are well-shuffled, while approximating connection input and output strengths. Histograms of correlation coefficients between the reconstructed network and the null models’ in (blue: 0.92 ± 0.02, mean ± s.d.) and out (red: 96 ± 0.01, mean ± s.d.) strength and connectivity matrix (grey: 9.1 × 10^{-4} ± 0.01, mean ± s.d.). e, Occurrences of three neuron connectivity motifs found in the reconstructed network between excitatory neuronal targets.
Extended Data Figure 8 | Cell bodies are functionally intermingled. 

**a, b.** Differences in peak orientation (a) and direction preference (b) between neuron pairs plotted against the distance between their cell bodies. Uniform distributions of functional versus spatial distance suggest a salt and pepper intermingling of neuronal cell bodies across functional properties.
Extended Data Figure 9 | Axons and dendrites are functionally intermingled at shorter length scales. Uniform functional diversity and prediction of connectivity at finer length scales suggest a salt-and-pepper intermingling of axons and dendrites. a–d. Same as Fig. 2c–f for $s = 1\,\mu m$. Significance tests: a, $P \approx 0$, permutation test, $n_{connected\,pairs} = 29$, $n_{unconnected\,pairs} = 1,951$; b, Between connected (red line) and unconnected pairs (blue line, $P < 0.05$, permutation test, $n_{connected\,pairs} = 29$, $n_{unconnected\,pairs} = 1,951$) or a model distribution based on potential synapse length (black line, $P < 0.01$, permutation test, $n_{connected\,pairs} = 29$, $n_{unconnected\,pairs} = 1,951$). Shaded regions, a, b, and error bars, d, represent bootstrapped standard error.
Extended Data Figure 10 | Connectivity is not predicted by residual signal correlation after removal of orientation preference. a–b, Example activity (ΔF/F individual trial time courses) for connected neurons from experiments varying direction, spatial and temporal frequencies of grating stimuli. a, Presynaptic cell (top row) and two of its postsynaptic partners’ (middle and bottom rows) for 3 spatial and 2 temporal frequencies and one orientation (orientation tuning was virtually identical). b, Presynaptic cell (top) and a postsynaptic deep layer apical dendrite’s (bottom) responses to 2 spatial and 2 temporal frequencies, and 2 directions, (again, orientation tuning was virtually identical). Grey window delineates time of stimulus presentation. Scale bars, 100% ΔF/F and 4 s. c, Cumulative distribution of signal correlations from simultaneously measured cells was significantly greater between connected than unconnected pairs (P < 0.01, permutation test, n_{connected pairs} = 10, n_{unconnected pairs} = 426) or a model distribution based on potential synaptic connectivity (P < 0.05, permutation test). d, After averaging over orientations, the cumulative distribution of signal correlations was similar between connected and unconnected pairs (P > 0.14, permutation test, n_{connected pairs} = 10, n_{unconnected pairs} = 426) and a model distribution based on potential synaptic connectivity (P > 0.25, permutation test, n_{connected pairs} = 10, n_{unconnected pairs} = 426). Shaded regions, c, d, represent bootstrapped standard error.