Cite as: A. Motta *et al.*, *Science* 10.1126/science.aay3134 (2019).

Dense connectomic reconstruction in layer 4 of the somatosensory cortex

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The dense circuit structure of mammalian cerebral cortex is still unknown. With developments in three-dimensional electron microscopy, the imaging of sizeable volumes of neuropil has become possible, but dense reconstruction of connectomes is the limiting step. We reconstructed a volume of ~500,000 µm³ from layer 4 of mouse barrel cortex, ~300 times larger than previous dense reconstructions from the mammalian cerebral cortex. The connectomic data allowed the extraction of inhibitory and excitatory neuron subtypes that were not predictable from geometric information. We quantified connectomic imprints consistent with Hebbian synaptic weight adaptation, which yielded upper bounds for the fraction of the circuit consistent with saturated long-term potentiation. These data establish an approach for connectomic phenotyping of local dense neuronal circuitry in the mammalian cortex.

The cerebral cortex of mammals houses an enormously complex intercellular interaction network implemented via neuronal processes that are long and thin, branching, and extremely densely packed. Early estimates reported an expected 4 km of axons and 400 m of dendrites compressed into a cubic millimeter of cortical tissue (*1*). This high packing density of cellular processes has made the locally dense mapping of neuronal networks in the cerebral cortex challenging.

So far, reconstructions of cortical tissue have been either sparse (*2–7*) or restricted to small volumes of up to 1,500 µm³ (*8–10*). Consequently the detailed network architecture of the cerebral cortex is unknown. Particular open questions are to what degree local neuronal circuits are explainable by geometric rules alone (*1*, *2*, *11–13*) – and on which spatial scales cortical connectivity is only explainable by innervation preferences beyond such geometric models (*5*, *8*, *9*, *14*, *15*). Similarly, while numerous cortical neuronal cell types have been described based on protein expression, morphology and electrophysiological characteristics (*16*), and these have been shown to have particular synaptic target patterns (*17*), the inverse question whether at the level of the dense cortical circuit, axons represent a continuum of synaptic preference or rather a set of distinct innervation paradigms which would allow for a purely connectomic cell type definition (as has been successful in the retina (*18*, *19*)) is still open. Next, at the level of synaptic input to the primary dendrites of cortical excitatory cells, it is not known whether the typically 3-10 primary dendrites of a cortical neuron that leave the cell body sample a homogeneous set of available excitatory and inhibitory synaptic inputs, or whether there is an enhanced heterogeneity of synaptic input composition, making it possible to exploit the numerous mechanisms that have been discussed for the non-linear integration of local synaptic inputs (*20–23*). Finally, while the change of synaptic weights in response to electrical and sensory stimulation has been widely studied (*24–28*), and connectomic data consistent with LTP has been described (*29*, *30*), the question what fraction of a given cortical circuit is plausibly shaped by processes related to Hebbian learning under undisturbed conditions is still unknown.

We used the dense connectomic reconstruction to quantitatively address these questions about the formational principles of a dense cortical circuit.

Results

We acquired a 3-dimensional EM dataset from a 28-day old mouse from upper layer 4 of primary somatosensory cortex (Fig. 1, A to D, likely located within a barrel, see supplementary materials) using serial block-face electron microscopy (SBEM, (*31*); dataset size: 61.8x94.8x92.6µm³, voxel size: 11.24x11.24x28nm³). For dense reconstruction (Fig. 1, E to H), we 3D-aligned the images and applied a sequence of automated analyses (SegEM, (*32*), SynEM (*33*), ConnectEM and TypeEM; Fig. 2, supplementary materials and methods, and table S2) followed by focused manual annotation (FocusEM). We reconstructed 89 neurons that had their cell body in the dataset (Fig. 1, E and F). These neurons constituted only 2.6% of the total path length (69 mm, Fig. 1G). To reconstruct

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axons, which constitute the majority of wiring in the dense circuit (1.79 m, 66.6%, Fig. 1H), we applied a scalable distributed annotation strategy, that identified locations of uncertainty in the automated reconstruction, which were then resolved by targeted manual annotation. To reduce the required manual annotation time, it was critical to obtain an automated reconstruction with low error rates; to use efficient algorithms for identifying locations for focused manual inspection (queries); and to minimize the time spent per user query. For this (Fig. 2A), we developed artificial intelligence-based algorithms that evaluated the EM image data and CNN-filtered versions of the image data in the surrounding of interjunctions between segmented pieces of neurites (Fig. 2B). Together with classifiers that computed the probability of volume segments to belong to an axon, a dendrite, a spine head or a glial process (using, among others, shape features, Fig. 2C), this allowed us to automatically connect parts of dendrites, attach spine heads to dendritic shafts (by a greedy step-wise agglomeration initiated at the spine head, Fig. 2D; 58.9% of spine heads unaffected by the dataset boundary were automatically attached), and reconstruct parts of axons. Similarly, synapses were automatically detected by evaluating pre- and postsynaptic volumes at neurite interfaces (Fig. 2E and figs. S2 to S5 (*33*); for shaft synapses, additional CNN-based classifiers for vesicle clouds and mitochondria were used). To manually correct remaining errors in axons (Fig. 2, F to H), we detected ending locations of automatically reconstructed axon pieces (Fig. 2F) and directed user queries to these locations. For this, we used an egocentric 3D image display mode ["flight mode," Fig. 2G (*34*)] and oriented the user annotation along the axis of the neurite for which a local annotation ("query") was requested (movie S2). Together with data pre-loading, this yielded a low-latency targeted neurite annotation in which single user queries took 29.4 s to resolve (traveled path length per query: 5.49 μm). These queries could be easily distributed among 87 annotators. Similarly, we detected locations of likely mergers between axons (Fig. 2H, "chiasmata") and directed user queries to reconnect the chiasma exits along actual axons. Thanks to this scalable annotation architecture, we obtained a dense reconstruction of 2.69 m of neuronal processes (Fig. 1, G and H) with a total investment of 3,981 human work hours – about 10 times faster than a recent dense reconstruction in the fly larval brain (*35*) (Fig. 2, I and J), about 20 times faster than the previous dense reconstruction from the mammalian retina (*18*), and about 25 times faster than the previous dense reconstruction from mammalian cortex (*9*) (Fig. 2, I and J). To quantify remaining reconstruction error rates in this dense neuropil reconstruction, we measured the remaining errors in a set of 10 randomly chosen axons and found 12.8 errors per millimeter of path length (of these 8.7 per millimeter continuity errors, see materials and methods). This is indistinguishable from the error rates previously found in fast human annotations (*18*, *34*, *36*).

We obtained a connectome (Fig. 3) between 34,221 presynaptic axonal processes and a total of 11,400 post-synaptic processes (6,979 by 3,719 connectivity matrix (Fig. 3E) when restricted to those pre- and postsynaptic neurites that established at least 10 synapses). Among the post-synaptic processes we classified n=169 apical dendrites (ADs) that traversed the dataset along the cortical axis without connection to one of the neuronal cell bodies in the dataset (Fig. 2A), 246 smooth dendrites (SDs, Fig. 2B), 80 somata, 116 axon initial segments (AIS, Fig. 2C), and 94 proximal dendritic trees connected to a soma in the dataset (PDs, movie S1; note that some of these neurons also had apical dendrites that were classified as PDs and not included in the AD definition above; see materials and methods and tables S1 and S2).

Connectomic definition of axon types

We investigated whether based solely on connectomic information (Fig. 3) we could extract the rules of subcellular innervation preference described for inhibitory axons in the mammalian cortex (*17*), and whether such synaptic target preference could also be found for excitatory axons. We first measured the preference of each axon for innervating dendritic spine heads versus dendritic shafts and other targets (Fig. 4, A and B), because in the mammalian cortex, most axons of inhibitory interneurons preferentially innervate the dendrites' shafts or neuronal somata (*17*), and most excitatory glutamatergic axons preferentially innervate the spine heads of dendrites (*1*). The fraction of primary spine synapses per axon (out of all synapses of that axon) accordingly allowed the identification of spine-preferring, likely excitatory axons with at least 50% primary spine innervations (n=5,894 axons) and shaft-preferring, likely inhibitory axons with less than 20% primary spine innervations (n=893 axons; 13.2% of all axons; for exceptions to this rule and control measurements, see supplementary materials and tables S1 and S2).

We then determined for each of the subcellular synaptic target classes defined above (Figs. 3 and 4C) the per-synapse innervation probability that would best explain whether an inhibitory axon establishes at least one synapse onto each of these targets. These inhibitory "single-hit" binomial innervation probabilities were 4.2% (Somata), 17.8% (PD), 4.9% (SD), 3.3% (AD), and 0.5% (AIS) (Fig. 4D). We then computed the expected distribution of synapses per axon made onto each target class assuming the double-hit, triple-hit, etc. innervation probabilities are the same as the probability to establish at least one synapse onto that target. When comparing these target distributions to the actually measured distributions of synapses per axon onto each target class (Fig. 4E), we found that inhibitory axons established enhanced preference for cell bodies ($p=2.4x10^{-34}$, n=893, one-sided Kolmogorov-

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Smirnov test), proximal dendrites ($p=6.0x10^{-77}$), apical dendrites ($p=2.5x10^{-4}$) and to a lesser degree for smooth dendrites ($p=1.7x10^{-3}$, table S1), but no enhanced preference for axon initial segments in L4 (p=0.648). AIS were synaptically innervated by 0.172 input synapses per μm AIS length, but these innervations were not made by axons with an enhanced preference for AIS, unlike in supra- and infragranular layers (*37*)).

When performing the same analysis for excitatory axons (Fig. 4F), we found clear target preference for apical dendrites ($p=2.5x10^{-34}$, Fig. 4F), for smooth dendrites ($p=7.6x10^{-25}$) and for proximal dendrites ($p=1.3x10^{-169}$). Thalamocortical axons [detected using the criteria reported in (*38*); see fig. S6 and materials and methods), to the contrary, showed indication of target preference for proximal dendrites ($p=2.5x10^{-31}$), but not for apical (p=0.019) or smooth dendrites (p=0.723). In order to determine the fraction of inhibitory and excitatory axons that had an unexpectedly high synaptic preference for one (or multiple) of the subcellular target classes, we used the false detection rate criterion used for the determination of significantly expressed genes (q value (*39*), see materials and methods) and obtained lower bounds on the fractions of axons in the tissue that are preferentially innervating the various subcellular target classes (Fig. 4G; at least 58.0% of inhibitory and 24.4% of excitatory axons). Inhibitory (Fig. 4H) but not excitatory axons (Fig. 4I) showed higher-order innervation preferences indicating that at the level of the dense cortical circuit, synaptic target preferences established by axons were not a continuum but allowed cell type classification without the need for measurements of neuronal morphology, electrical activity, protein expression or transcription levels.

Geometric sources of synaptic innervations

Could these local connectivity rules have been derived solely from the geometry of axons and dendrites? We first quantified the overall relation between the spatial distribution of axons and dendrites and the establishment of synapses between them (Fig. 5). One paradigm, originally proposed by Peters (*11*), states that thalamocortical axons entering a certain cortical tissue volume would sample the available cortical dendrites for synaptic innervation according to their relative prevalence in the tissue (*1*). This model (Fig. 5A) predicted the thalamocortical innervation of most cortical dendrites rather well with the exception of smooth dendrites (an exception reported by (*14*)) and the enhanced TC innervation of proximal dendrites of layer 4 cells (*21*). When applied to corticortical excitatory and inhibitory axons (Fig. 5A), we found that this model predicted excitatory innervation of most spiny dendrites rather well, but again failed to predict innervation of smooth dendrites and the proximal bias of inhibitory synapses. Since this model (which has been most widely used for circuit inference, (*2*, *12*)) implicitly accounts for the density of synapses along the presynaptic axons, it was capable of capturing the increased synapse density of thalamocortical axons (Fig. 5A). A simpler variant of Peters' model (*1*, *15*) (Fig. 5B), which uses the density of pre- and postsynaptic path length as basis for the synaptic innervation prediction, failed at predicting the thalamocortical innervation, but captured the corticocortical innervation of spiny dendrites (Fig. 5B). We then analyzed whether a "Peters'" model normalized for postsynaptic synapse density (Fig. 5C) would better capture synaptic innervation since it implicitly accounts for the postsynaptic synapse density, and found that in fact, the dendritic model was a far better predictor of synaptic innervation (compare Fig. 5, C and B). This showed that smooth dendrites and apical dendrites sampled synaptic input according to the relative path length of the presynaptic axons (Fig. 5C). We then investigated whether a Peters' model accounting for pre- *and* postsynaptic synapse densities would improve the innervation prediction (Fig. 5D). In this model, both the output and the input of cortical excitatory neurites were properly predicted, but the suppressed innervation of smooth and apical dendrites by thalamocortical axons and the proximal bias of inhibitory axons was not. Notably, none of the Peters' models could account for this proximal bias of inhibitory synapses [Fig. 5D; for other failures of Peters' predictions see for example (*3*, *6*, *8*, *9*)].

More recently, the Peters' model has been investigated for the close proximity between axons and dendrites on the scale of few micrometers (*8*, *9*) and concluded poor (*8*) or absent (*9*) geometric predictability of synaptic innervation. We used our larger dense reconstruction to investigate the geometric prediction over a substantially broader spatial scale to about 30μm, and accounted for inhibitory, excitatory axons and postsynaptic target types (Fig. 5, E to H). We measured whether the postsynaptic membrane surface available within a certain radius r_{pred} around a given axon (Fig. 5E) would be a predictor of synaptic innervation for that given axon. We measured the available membrane surface belonging to the 5 subcellular target classes around all 6,979 axons (Fig. 5F) and used a linear multinomial regression model to predict synaptic innervation from this data (Fig. 5G). Then we computed the coefficient of determination (R^2) reporting the fraction of axonal synaptic innervation variance that could be explained purely based on the geometrical information (Fig. 5H; for details, see supplementary materials and materials and methods for details). In fact, for small spatial scales of 1-5 μm, the membrane surface available around an axon was a rather good predictor of synaptic innervation from excitatory axons (range 16-90%, Fig. 5H; less so for inhibitory axons: 23-79%).

Would this imply that axonal and dendritic proximity at the single-axon level can be used to infer synaptic connectivity in the cortex (*13*)? We found that for the spatial alignment

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scales that can be achieved in light-microscopy based neuron reconstructions from multiple animals (10-20 μm), predictability dropped dramatically (Fig. 5H), making circuit inference by an emulation of growth processes based on light-microscopically aligned data (*13*, *40*) implausible.

Subcellular synapse placement

We used our dense segmentation to study the spatial distribution of synapses along somata and dendrites in the cortical neuropil. The density of thalamocortical synapses had a substantial dependence on cortex depth (Fig. 6, A to D): the absolute density of TC synapses in the volume increased by about 93% over 50 μm cortex depth (Fig. 6, A and B; the TC excitatory synapse fraction TC/(TC+CC) increased by 82.6%, corresponding to an absolute increase in the TC synapse fraction of 5.8% per 50 μm cortex depth, Fig. 6D). This gradient was consistent with light-microscopic analyses of TC synapses indicating a decrease of TC synapse density from lower to upper L4 (*41*) which is most substantial when analyzed at the level of single VPM axons (*41*). Neither the inhibitory nor the corticocortical excitatory synapse densities showed a comparable spatial profile (Fig. 6C).

How was the synaptic TC gradient mapped onto the dendrites of L4 neurons along the cortex axis (Fig. 6, E to G)? One possibility was that the TC synapse gradient was used to enhance the variability of synaptic input composition between different primary dendrites of the L4 neurons such that a neuron's dendrites pointing upwards toward the pia would sample relatively less TC input than dendrites pointing toward the white matter. Alternatively, mechanisms to establish synaptic target preference (as those reported in Fig. 4) could be used to counterbalance this synaptic gradient and equilibrate the synaptic input fractions on the differently oriented dendrites. Our analysis showed that in fact, even at the level of single primary dendrites, TC input fractions were 1.28-fold higher for dendrites pointing upwards toward the cortical surface vs downwards toward the white matter (Fig. 6, F and G; TC input fractions of each dendrite were corrected for the entire neuron's TC input fraction for this analysis, see materials and methods). We then asked whether this differential composition of the excitatory inputs was accompanied by different compositions of the inhibitory input synapses (Fig. 6, H to L). We found that the fraction of TC input to a neuron's dendrites was anticorrelated to the fraction of inhibitory synapses that originated from apical-dendrite preferring inhibitory axons (Figs. 6I and 4) - both at the level of the input to L4 neurons and at the level of single primary dendrites of L4 neurons (Fig. 6, I and J). The effect was absent for all other synapse classes, most notably the soma-preferring inhibitory axons (Fig. 6K; see Discussion).

Connectomic mapping of the plasticity-consistent circuit fraction

The concept of Hebbian plasticity, thought to be at the core of experience-dependent changes of synaptic weights in the brain, makes predictions about the temporal evolution of synaptic weights in multiple synaptic contacts between the same pre- and postsynaptic neurons (joint synapses, Fig. 7, A and B): Because Hebbian synaptic plasticity is dependent on the electrical activity of the pre- and postsynaptic neurons, which in a first approximation can be assumed to be similar at joint synapses, long-term potentiation (LTP) predicts joint synapses to become stronger and relatively more similar in weight (especially if synaptic weight saturates); and long-term depression (LTD) predicts joint synapses to become weaker and relatively more dissimilar in weight (but more similar if synaptic weights saturate, Fig. 7A). With this, models of LTP and LTD make particular predictions about the temporal evolution of joint synaptic weights, and the mapping of synaptic weights and synaptic weight similarity in the connectome allows the quantification of upper bounds on the fraction of the circuit that can have undergone such particular patterns of weight change prior to the connectomic experiment (we denote those synapse pairs for which such patterns of weight change occurred to a sufficient degree as "having undergone LTP/LTD"; see Discussion).

We set out to leverage our large connectomic dataset (n=5,290 of excitatory joint synaptic pairs onto spines, Fig. 7C) to map the relation between synaptic size and synaptic size similarity in joint synapse pairs [Fig. 7E; for visualization, the figure panels report relative synaptic size *dis*similarity on the x-axis; for the utilization of axon-spine interface area (Fig. 7D) as indicator of synaptic weight (*42*, *43*)]. This data would allow us to determine upper bounds on the plasticity-consistent fraction of the circuit, beyond the previous finding that in joint synapse pairs, synaptic size is more similar than for randomly shuffled synapse pairs (*9*, *29*, *30*, *44*).

Synaptic size similarity in joint synapse pairs showed a broad distribution (Fig. 7E). When comparing this distribution to the synaptic size and synaptic size similarity distribution obtained from a random assignment of the same synapses into "random pairs" (Fig. 7F and fig. S7, C and D), we observed that the population of over-similar synapse pairs (Fig. 7F) is split into a region of over-similar and large synapses (mean synaptic size 0.23-1.19 μm^2; in this region, 16-20% of all joint synapse pairs are found; the above-random synapse pairs constitute 3.6-3.9% of all joint synapse pairs, see fig. S7, C and D, and materials and methods for details of the region definition and statistics), and over-similar and small synapses (mean synaptic size 0.06-0.2 μm^2; in this region, 15-19% of all joint synapse pairs were found; 3.0-3.4% of all joint synapse pairs were above-random in this region). With this we obtained upper bounds on the fraction of the circuit that

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can have undergone LTP and LTD with weight saturation (compare Fig. 7, F and A).

To what degree was the observed synaptic weight similarity a result of subtypes of neurons establishing differently sized synapses? While the quantification of the upper bounds of the plasticity-consistent circuit fraction would remain unaffected, we could use this more detailed analysis to understand whether the plasticity-consistent circuit fractions were specific to types of neuronal connections.

First, we considered the possibility that certain presynaptic cell types made consistently larger or consistently smaller synapses (Fig. 7G). In this case, the distribution of synaptic weight similarity for same-axon different-dendrite synapse pairs would also show a bias toward more similarly sized synapses. However, we found no such evidence (Fig. 7H), excluding cell-type specific synapse size of either presynaptic (axonal) or postsynaptic (dendritic) origin as the cause of the observed over-similar synapse pairs.

Then, we separated those connections established by TC axons from those made by the remaining excitatory (i.e., CC) axons (Fig. 7I and fig. S7, A and B). We found an excess of over-similar synapse pairs in the TC connections, as well, with 6-15% of pairs found in a region of overly similar and large synapses (i.e., upper bound of 15% on LTP). The region of overly similar and small synapse pairs, however, only comprised 2-7% of joint synapse pairs. This remaining number of overly similar small synapse pairs could in fact be induced by the overly similar and large synapse pairs (see supplementary materials). At 28 days of age, about 3 weeks after the proposed critical period during which LTP can be induced in thalamocortical connections (*45*, *46*), a fraction of up to 15% of joint synapse pairs was still consistent with previous episodes of LTP that led to stabilized potentiated synapse pairs at dendritic spines (*47*, *48*), but 85% were not.

Repeating these analyses for other combinations of pre- and postsynaptic neurite types (Fig. 7J), we found upper bounds for LTP and LTD of 10-20%. For each of these subtype-specific connections we could then again analyze whether any purely presynaptic or purely postsynaptic subtype within the already type-selected connections (corresponding to squares in the table of Fig. 7J) could be the cause of the observed synapse similarity. For example, the connections from corticortical axons onto spiny L4 neurons (*49*) showed no evidence for presynaptic axonal subtypes yielding over-similar synapses (Fig. 7K; for additional controls of these findings, see supplementary materials; fig. S7, A and B; and table S1).

Together, these results provided a first quantitative upper bound on the fraction of the circuit consistent with previous episodes of saturated Hebbian synaptic plasticity leading to strengthening or weakening of synapses (a "connectomic fingerprint" of the maximum possible plasticity fraction of the circuit), and excluded obvious cell-type based connection strength differences as the origin of these observations. Because these results were obtained from brains of un-trained animals, and not as a result of electrical or other stimulation ("plasticity induction"), these data may represent an unbiased screening of upper bounds of plasticity traces in local cortical circuits, for which the dense connectomic mapping was essential.

Discussion

Using FocusEM, we obtained the first dense circuit reconstruction from the mammalian cerebral cortex at a scale that allowed the analysis of axonal rules of subcellular innervation - about 300 times larger than previous dense reconstructions from cortex (*9*). Inhibitory axonal types preferentially innervating certain postsynaptic subcellular compartments could be defined solely based on connectomic information (Figs. 3 and 4). In addition to inhibitory axons, a fraction of excitatory axons also exhibited such subcellular innervation preferences (Fig. 4). The geometrical arrangement of axons and dendrites explained only a moderate fraction of synaptic innervation, revoking random models of cortical wiring (Fig. 5). A substantial thalamocortical synapse gradient in L4 gave rise to an enhanced heterogeneity of synaptic input composition at the level of single cortical dendrites (Fig. 6), which was accompanied by a reduced innervation from apical dendrite-preferring inhibitory inputs. The consistency of synapse size between pairs of axons and dendrites signified fractions of the circuit consistent with saturated synaptic plasticity, placing an upper bound on the "learned" fraction of the circuit (Fig. 7). Together, FocusEM allowed the dense mapping of circuits in the cerebral cortex at a throughput that enables connectomic screening.

Synaptic input composition along L4 dendrites

Our finding of a covariation of enhanced TC inputs to L4 excitatory cells with reduced direct inhibitory input from apical-dendrite preferring interneurons (Fig. 6, H to K) could be interpreted in the context of a disinhibitory circuit described before (*50*, *51*). Taking into account the preferential targeting of apical dendrites and of soma-preferring PV-positive interneurons by SST-positive INs, this could imply that SST-IN based disinhibition can enhance TC input by silencing perisomatic PV inputs recruited via feedforward inhibition (*52*), and concomitantly reducing the direct inhibitory component from SST INs. In any case this finding of per-dendrite input variation points to a circuit configuration in which TC input variability is enhanced between neurons of the same excitatory type in cortical layer 4, and furthermore provides evidence for a per-dendrite synaptic input composition of enhanced heterogeneity.

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Connectomic traces of plasticity

We interpreted the joint synapse data (Fig. 7) in terms of upper bounds of synapse pairs that can have undergone certain models of plasticity. While this analysis detects those synapse pairs that were exposed to saturating plasticity (i.e., the possible plasticity event led to a final weight state of both synapses), an alternative interpretation is a dynamic circuit in which at any given point in time, only a fraction of synapses has expressed saturated plasticity, while other (or all) synapses are in the process of undergoing plastic changes. We expect that such more elaborate plasticity models of entire circuits will make testable predictions that are accessible by connectomic snapshot experiments as shown here.

Outlook

The presented methods and results open the path to the connectomic screening of mammalian tissue from various cortices, layers, species, developmental stages, sensory experience and disease conditions. The fact that even a small piece of mammalian cortical neuropil contains a high density of relevant information, so rich as to allow the extraction of possible connectomic signatures of the "learnedness" of the circuit, makes this approach a promising endeavor for the study of the structural setup of mammalian nervous systems.

Materials and Methods

Animal experiments

A wild-type (C57BL/6) male mouse was transcardially perfused at postnatal day 28 under isoflurane anesthesia using a solution of 2.5% paraformaldehyde and 1.25% glutaraldehyde (pH 7.4) following the protocol in (*53*). All procedures followed the animal experiment regulations of the Max Planck Society and were approved by the local animal welfare authorities (Regierungspräsidien Oberbayern and Darmstadt).

Tissue sampling and staining

The fixated brain was removed from the skull after 48h of fixation and sliced coronally using a vibratome. Two samples were extracted using a 1 mm biopsy punch (Integra Miltex, Plainsboro, NJ) from a 1 mm thick slice at 5 mm distance from the front of the brain targeted to layer 4 in somatosensory cortex of the right hemisphere. The corresponding tissue from the left hemisphere was further sliced into 70 μm-thick slices followed by cytochrome oxidase staining indicating the location of the coronal slice to be in barrel cortex.

Afterwards the extracted tissue was stained as in (*53*). Briefly, the tissue was immersed in a reduced Osmium tetroxide solution (2% OsO_4, 0.15 M CB, 2.5 M KFeCN) followed by a 1% Thiocarbohydrazide step and a 2% OsO_4 step for amplification. After an overnight wash, the sample was further incubated with 1.5% Uranyl Acetate solution and a 0.02 M Lead(II) Nitrate solution. The sample was dehydrated with Propylenoxide and EtOH, embedded in Epon Hard (Serva Electrophoresis GmbH, Germany) and hardened for 48 hours at 60°C.

3D EM experiment

The embedded sample was placed on an aluminum stub and trimmed such that on all four sides of the sample the tissue was directly exposed. The sides of the sample were covered with gold in a sputter coater (Leica Microsystems, Wetzlar, Germany). Then, the sample was placed into a SBEM setup ((*31*), Magellan scanning electron microscope, FEI Company, Hillsboro, OR, equipped with a custom-built microtome courtesy of W Denk). The sample was oriented so that the radial cortex axis was in the cutting plane. The transition between L4 and L5A was identified in overview EM images by the sudden drop in soma density between the two layers (Fig. 1C). A region of size 96 μm × 64 μm within L4 was selected for imaging using a 3 by 3 image mosaic, a pixel size of 11.24 × 11.24 nm^2, image acquisition rate of 10 MHz, nominal beam current of 3.2 nA (thus a nominal electron dose of 15.8 e^-/nm^2), acceleration voltage of 2.5 kV and nominal cutting thickness of 28 nm. The effective data rate including overhead time spent during motor movements for cutting and tiling was 0.9 MB/s. 3,420 image planes were acquired, yielding 194 GB of data.

Image alignment

After 3D EM dataset acquisition, all images were inspected manually and marked for imaging artifacts caused by debris present on the sample surface during imaging. Images with debris artifacts were replaced by the images at the same mosaic position from the previous or subsequent plane. First, rigid translation-only alignment was performed based on the procedures in (*53*). The following modifications were applied: When shift vectors were obtained that yielded offsets of more than 100 pixels, these errors were iteratively corrected by manually reducing the weight of the corresponding entry in the least-square relaxation by a factor of 1000 until the highest remaining residual error was less than 10 pixels. Shift calculation of subsequent images in cutting direction was found to be the most reliable measurement and was therefore weighted 3-fold in the weighted least-square relaxation. The resulting shift vectors were applied (shift by integer voxel numbers) and the 3D image data was written in KNOSSOS format (*34*, *36*). For further improvement, sub-image alignment was applied (see Supplementary Methods).

Methods description for software code

All routines described in the following are available as software at https://gitlab.mpcdf.mpg.de/connectomics/L4dense, which is the relevant reference for the exact sequence of processing steps applied. The following descriptions and the more detailed ones in the Supplementary Methods are aimed

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at pointing to the key algorithmic steps rather than enumerating all detailed computations.

Workflow for dense circuit reconstruction

The workflow for volume reconstruction of the acquired 3D EM volume (Fig. 2 and fig. S1) was as follows. We first detected blood vessels and cell bodies using automated heuristics, and then processed the remaining image volume using machine-learning-based image segmentation [convolutional neural network (CNN) and watershed as described in SegEM, (*32*)]. The result of this processing were 15 million volume segments corresponding to pieces of axons, dendrites and somata (volume: 0.0295 ± 0.3846 μm^3; mean ± s.d.). We then constructed the neighborhood graph between all these volume segments and computed the properties of interfaces between directly adjacent volume segments. Based on these features we trained a connectivity classifier (ConnectEM, Fig. 2, A and B) to determine whether two segments should be connected (along an axon or a dendrite or a glial cell) or whether they should be disconnected. Using the SynEM classifier (*33*), we determined whether an interface between two disconnected processes corresponded to a chemical synapse, and if so, which was the pre- and which the postsynaptic neurite segment (see below for more details). We furthermore trained a set of classifiers (TypeEM, Fig. 2C) to compute for each volume segment the probability to be part of an axon, a dendrite, a spine head or a glia cell (precision and recall were 91.8%, 92.9% for axons, 95.3%, 90.7% for dendrites, 97.2%, 85.9% for astrocytes, and 92.6%, 94.4% for spine heads, respectively, see table S2).

Cell body-based neuron reconstruction

Then, we reconstructed those neurons, which had their cell bodies in the tissue volume (Fig. 1, E and F, cell gallery in movie S1; n=125 cell bodies, of these 97 neuronal, of these 89 reconstructed with dendrites in the dataset). For this, we used a set of simple growth rules for automatically connecting neurite pieces based on the segment-to-segment neighborhood graph and the connectivity and neurite type classifiers (fig. S1, "automated agglomeration," see Suppl. Methods). As a result, we obtained fully automated reconstructions of the neuron's soma and dendritic processes. Notably with a minimal additional manual correction investment of 9.7 hours for 89 cells (54.5 mm dendritic and 2.1 mm axonal path length), the dendritic shafts of these neurons could be reconstructed without merge errors, but 37 remaining split errors, at 87.3% dendritic length recall (table S2). This reconstruction efficiency compares favorably to recent reports of automated segmentation of neurons in 3D EM data from the bird brain obtained at about 2-fold higher imaging resolution (*54*), which reports soma-based neuron reconstruction at an error rate of beyond 100 errors per 66 mm dendritic shafts at lower (68%) dendritic length recall, with a similar resource investment (see Suppl. Methods).

In addition to the dendritic shafts, the dendritic spines constitute a major fraction of the dendritic path length in cortical neuropil (Fig. 1G). Using our spine head classifier (part of the TypeEM classifiers, Fig. 2C), we found 415,797 spine heads in the tissue volume i. e. density of 0.784 per μm^3 (0.98 per μm^3 of neuropil, when excluding somata and blood vessels). In order to connect these to the corresponding dendritic shafts we trained a spine neck continuity algorithm that was able to automatically attach 58.9% of these spines (evaluated in the center of the dataset, at least 10 μm from the dataset border) yielding a dendritic spine density of 0.672 per μm dendritic shaft length (comparable to spine densities in the bird brain, (*55*)). However in mammalian cerebral cortex, the density of spines along dendrites is even higher (at least 1 per μm dendritic shaft length). The remaining spine heads were then attached to their dendritic shafts by seeding manual reconstructions at the spine heads and asking annotators to continue along the spine necks to the dendritic shafts. This annotation was carried out in the "orthogonal mode" configuration of webKnossos (*34*) in which the annotator viewed 3 orthogonal image planes to decide where to continue the respective spine neck (as in KNOSSOS (*36*)). The annotation of all remaining spine necks consumed an additional 900 hours of human work for the attachment of 98,221 spines, resulting in a final overall spine density of 0.959 per μm dendritic shaft length.

Dense tissue reconstruction

The reconstruction of neurons starting from their cell bodies was however not the main challenge. Rather, the remaining processes, that is axons and dendrites not connected to a cell body within the dataset and densely packed in the tissue, constitute about 97% of the total neuronal path length in this volume of cortex (Fig. 1G). To reconstruct this vast majority of neurites (Fig. 1H), we first used our connectivity and neurite type classifiers (ConnectEM and TypeEM, Fig. 2) to combine neurite pieces into larger dendritic and axonal agglomerates ("automated agglomeration," fig. S1 and supplementary materials and methods). Then, we took those agglomerates that had a length of at least 5 μm (n=74,074 axon agglomerates), detected their endings that were not at the dataset border and directed focused human annotation to these endings ("queries," Fig. 2, F and G).

For human annotation, we used an egocentric directed 3D image data view ("flight mode" in webKnossos), which we had previously found to provide maximized human reconstruction speed along axons and dendrites in cortex (*34*). Here, however, instead of asking human annotators to reconstruct entire dendrites or axons, we only queried their judgement at the endings of automatically reconstructed neurite

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parts. To make these queries efficient, we made three additions to webKnossos: We oriented the user along the estimated direction of the neurite at its ending, reducing the time the user needs to orient within the 3D brain tissue; we dynamically stopped the user's flight along the axon or dendrite whenever another of the already reconstructed neurite agglomerates had been reached; and we pre-loaded the next query while the user was annotating (Fig. 2, F and G). Movie S2 illustrates this annotation process for cases of splits and mergers, respectively. Note that the user was able to switch quickly to the next query, and based on its 3D orientation spent little time orienting in the tissue at the novel location.

With this, the average user interaction time was 21.3 ± 36.1 s per query, corresponding to an average of 5.5 ± 8.8 μm traveled per query. In total, 242,271 axon ending queries consumed 1,978 paid out work hours (i.e., including all overheads, 29.4 s per query).

However, we had to account for a second kind of reconstruction error, so-called mergers, which can originate from the original segmentation, the agglomeration procedure, or erroneous flight paths from human queries (Fig. 2H). In order to detect such mergers, we started with the notion that most of these merger locations will yield a peculiar geometrical arrangement of a 4-fold neurite intersection once all neurite breaks have been corrected ("chiasma," Fig. 2H). Since such chiasmatic configurations occur rarely in branching neurites, we directed human focused annotation to these locations. First, we automatically detected these chiasmatic locations using a simple heuristic to detect locations at which axon-centered spheres intersected more than three times with the axon (Fig. 2H, n=55,161 chiasmata; for approaches to detect such locations by machine learning, see (*56*, *57*)). Then, we positioned the user queries at a certain distance from the chiasma location, pointing inward (Fig. 2H) and then used a set of case distinctions to query a given chiasma until its configuration had been resolved (see Methods for details). Chiasma annotation consumed an additional 1,132 work hours (note that the detection of endings and chiasmata was iterated 8 times for axons, see Methods, and that in a final step we also detected and queried 3-fold neurite configurations to remove remaining mergers).

Synapse detection, types of postsynaptic targets and connectome reconstruction

Given the reconstructed pre- and postsynaptic neurites in the tissue volume, we then went on to extract their connectome. For this we used SynEM (*33*) to detect synapses between the axonal presynaptic processes and the postsynaptic neurites. For synapses between axons and spine heads (for non-spine synapses, improvements to SynEM were made to enhance precision and recall, see Methods).

In particular, we trained a dedicated interface classifier for shaft synapses using training data containing only shaft and soma synapses. Furthermore this classifier also used four additional texture filters compared to SynEM in (*33*), which originated from the voxelwise predictions of a multi-class CNN trained on synaptic junctions, vesicle clouds, mitochondria and a background class.

Since we were interested in analyzing the subcellular specificity of neuronal innervation, we had to also classify which of the post-synaptic membranes belong to cell bodies; to classify spiny dendrites as belonging to excitatory cells, smooth dendrites belonging to interneurons; and to detect axon initial segments and those dendrites that were likely apical dendrites of neurons located in deeper cortical layers. We developed semi-automated heuristics to detect these subcellular compartments (Fig. 3, A to D; see supplementary materials and methods for details).

Definition of excitatory and inhibitory axons

We used the fraction of primary spine synapses per axon (out of all synapses of that axon; only axons with at least 5 μm path length and at least 10 synapses were analyzed), which had a peak at about 80% (Fig. 4, A and B), to identify spine-preferring, likely excitatory axons with at least 50% primary spine innervations. Similarly, we identified shaft-preferring, likely inhibitory axons with less than 20% primary spine innervations. Together this yielded 6,449 axons with clear shaft or spine preferences. For the remaining n=528 axons with primary spine innervations above 20% and below 50%, we first wanted to exclude remaining mergers between excitatory and inhibitory axons (that would yield intermediate spine innervation rates) and split these axons at possible merger locations (at least 3-fold intersections). Of these, 338 now had at least 10 synapses and spine innervation rates below 20% or above 50%. The remaining n=192 axons (2.75% of all axons with at least 10 synapses) were not included in the following analyses. This together yielded n=5,894 excitatory and n=893 inhibitory axons in our data. For additional controls, see supplementary materials.

Thalamocortical axons were defined following parameters described by (*38*), see Supplementary Methods.

Analysis of subcellular synaptic target preference

First, we assumed that all synapses of a given axon class have the same probability to innervate a particular postsynaptic target class (as above). We then inferred this single-hit innervation rate for each axon- and postsynaptic target-class by determining the probability which best explains whether or not an axon innervated the target class under a binomial model. The optimized binomial model was then used together with the measured number of synapses of each axon to calculate the expected distribution of target innervation rates. A one-sided Kolmogorov-Smirnov was used to test for

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the existence of a subpopulation with increased target innervation rate. To identify those axons that innervated a given target class beyond chance (Fig. 4G), we computed the probability $p^{(t)}_{meas,i,k}$ of finding at least the measured fraction of synapses onto target t for each axon i from axon class k. The p-values were also calculated for the expected distribution of target innervation rates and combined with $p^{(t)}_{meas,i,k}$ to estimate the p-value threshold $p\hat{}^{(t)}_{k}$ at which the false discovery rate q (*39*) crosses 20%. 80% of the axons with $p^{(t)}_{meas,i,k} < p\hat{}^{(t)}_{k}$ are innervating target t with a rate above the single-hit innervation probability and are thus called to be t-preferring.

For the analysis of 2nd order innervation preference (Fig. 4, H and I), we reported the fraction of synapses onto target τ by t-preferring axons of class k after removal of synapses onto t. This innervation rate was compared against the fraction of synapses onto target τ by all axons of class k.

Geometrical predictability analysis

Peters' rule (*1*) stipulates that synapses between classes of axons and dendrites are established in proportion to the prevalence of these classes. The first variant of Peters' rule considered (Fig. 5A) makes the prediction that the fraction of synapses from axon class A onto target class T is the product of p_A and q_T, where p_A is the proportion of axonal path length made up by class A, and q_T is the proportion of dendritic path length (excluding spines) made up by class T. The measured synapse fractions were compared against the predictions by calculating the ratio of observed to predicted synapse fractions.

This formulation predicts that a fraction q_T of the synapses is established onto target T, independent of the axon class (Fig. 5B). Similarly, all dendrite types are expected to receive a fraction p_A of their synapses from axon class A (Fig. 5C). Deviations from these predictions were quantified by normalizing the class connectome row- and column-wise and by calculating the ratio of observed synapse fractions to the marginal distributions along the columns and rows, respectively.

To assess the effect of incorporating explicit knowledge about the synapse densities of different axon and dendrite classes, a second variant of Peters' rule (Fig. 5D) was considered in which the predicted synapse fraction from axon class A onto target class T is the product of p'_A and q'_T, with p'_A and q'_T being the overall fractions of synapses originating from A and innervating T, respectively.

How much additional information about the neuropil composition around an axon helps to predict its postsynaptic targets was assessed as follows: For each axon we determined the total surface area of the target classes that were contained within the cylinder of radius r_{pred} around the axon (Fig. 5E) and compared it to the actually innervated target fraction of each axon (Fig. 5, E and F). We then analyzed the correlation between the availability of the target surfaces and the actually established synapses on these target classes (Fig. 5G).

To obtain an overall predictability quantification, we then computed the coefficient of determination (R^2) using the following model: For all axons of given type, we used the fraction of target innervations and fractional surface availabilities in a given surround of radius r_{pred} to find the optimal multivariate linear regression parameters. To estimate best-case geometric predictability, we then calculated the R^2 value as 1 minus the ratio of the residuals to synaptic variance on the same axons used for parameter optimization, while correcting for the variance introduced by the finite number of synapses per axon. Accordingly, we used the axons' fractional surface availabilities within r_{pred} and absolute synapse numbers to calculate the expected binomial variance, and subtracted it from the squared residuals.

This analysis made several assumptions that were in favor of a geometrical explanation of synaptic innervation [therefore the conclusions about a minimal predictability (Fig. 5H) are still upper bound estimates]: it was assumed that the number of synapses for a given axon was already known; in most settings, only average synapse rates are known for a given circuit; it also assumed that a precise knowledge of the axonal trajectory and the surrounding target surface fractions were available; again, this is usually only available as an average on the scale of r_{pred} of several 10's of micrometers.

To relax the assumption of complete knowledge about target availabilities, we repeated the above R^2 analysis for a model in which the predicted fractional innervation of a target is the fractional surface availability of that target.

The employed computational routine can be found at https://gitlab.mpcdf.mpg.de/connectomics/L4dense_in+connectEM/+Connectome/plotGeometricPredictability.m.

Synapse-size consistency analysis

To determine the consistency of primary spine synapses between a given axon-dendrite pair, we calculated the axon-spine interface area (ASI, (*33*, *43*)) of a synapse as the total contact area between the corresponding axon and spine head agglomerates. For axon-dendrite pairs connected by exactly two primary spine synapses, we then calculated the coefficient of variation (CV) of the ASI areas by $CV = 2^{1/2} (ASI_1 - ASI_2) / (ASI_1 + ASI_2)$ with ASI_1 and ASI_2 being the larger and smaller of the two ASI areas, respectively. To avoid false same-axon same-dendrite (AADD) pairs caused by remaining merge errors in the axon reconstruction, this analysis was performed only after splitting axons at their branch points. The measured distribution of CV values was compared against the CV values obtained by randomly drawing pairs from all AADD synapses and against the CV values of observed synapse pairs from the same axon onto different dendrites (AADd), from different axons onto the same dendrite

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(AaDD), and from different axons onto different dendrites (AaDd; Fig. 7H). To test whether AADD primary spine synapse pairs are more similar in size than pairs in the control conditions, a one-sided Kolmogorov-Smirnov test was used. We calculated the decimal logarithm of the average ASI area (in μm^2) and the CV of the ASI areas of each synapse pair to map the size-similarity plane (Fig. 7, F and I). The kernel density estimate of the observed distribution was compared against the distribution expected from random pairs (5,000 Monte Carlo samples; fig. S7C) to identify statistically significantly overrepresented regions. Contour lines show the intersection of the significance regions for p-value thresholds of 0.5% up to 5% (Fig. 7, E, F, I, and fig. S7, C and D) with the convex hull around the set of all data points. The fraction of data points contained within a contour was used as upper bound on the fraction of connections consistent with saturated Hebbian plasticity (Fig. 7J).

Statistical methods

The following statistical tests were performed (in order of presentation in the figures):

The existence of axon subpopulation with unexpectedly high synapse rate onto a given target class was tested using the one-sided Kolmogorov-Smirnov test (Fig. 4, E and F). Axons belonging to a given target-preference class were identified based on the false detection rate criterion [q=20% (*39*)] (Fig. 4G).

The degree to which synaptic variance is explainable by geometry-based models was evaluated using the coefficient of determination (R^2) (Fig. 5H). Binomial variance was corrected for by subtracting the surface fraction-based expected binomial variance from the squared residuals.

F-tests were used to evaluate synaptic gradients as function of cortical depth (Fig. 6, B and D) or dendritic orientation (Fig. 6, F and G). For correlation of TC input fraction with other synaptic input fractions along dendrites, inhibitory input fraction and 7 target-preferential inhibitory and excitatory synapse types were tested. AD-preferring inhibitory synapses were the only with significant and substantial correlation (Pearson correlation after Bonferroni correction for n=8 multiple tests). The correlation was also significant at the soma-level (Pearson correlation). Both correlations were also significant using Spearman rank correlation.

The four variants of Peters' rule (Fig. 5, A to D) were compared using a likelihood-ratio test based on the following multinomial model: It was assumed that the pre- and postsynaptic classes of each synapse in the connectome were sampled either following the path length fractions of these classes (p_A and q_T) or following the product of the path length and a class-specific likelihood-maximizing relative synapse density. Wilk's theorem was used to compute the corresponding p-values.

To test whether the axon-spine interface areas of a given spine synapse pair configuration were more similar than randomly sampled pairs, a one-sided Kolmogorov-Smirnov test was used (Fig. 7, H and K).

REFERENCES AND NOTES

1. V. Braitenberg, A. Schüz, *Cortex: Statistics and Geometry of Neuronal Connectivity*. (Springer, 1998).
2. J. Lübke, A. Roth, D. Feldmeyer, B. Sakmann, Morphometric analysis of the columnar innervation domain of neurons connecting layer 4 and layer 2/3 of juvenile rat barrel cortex. *Cereb. Cortex* **13**, 1051–1063 (2003). doi:10.1093/cercor/13.10.1051 Medline
3. N. M. da Costa, K. A. Martin, Selective targeting of the dendrites of corticothalamic cells by thalamic afferents in area 17 of the cat. *J. Neurosci.* **29**, 13919–13928 (2009). doi:10.1523/JNEUROSCI.2785-09.2009 Medline
4. D. D. Bock, W.-C. A. Lee, A. M. Kerlin, M. L. Andermann, G. Hood, A. W. Wetzel, S. Yurgenson, E. R. Soucy, H. S. Kim, R. C. Reid, Network anatomy and in vivo physiology of visual cortical neurons. *Nature* **471**, 177–182 (2011). doi:10.1038/nature09802 Medline
5. W. C. Lee, V. Bonin, M. Reed, B. J. Graham, G. Hood, K. Glattfelder, R. C. Reid, Anatomy and function of an excitatory network in the visual cortex. *Nature* **532**, 370–374 (2016). doi:10.1038/nature17192 Medline
6. H. Schmidt, A. Gour, J. Straehle, K. M. Boergens, M. Brecht, M. Helmstaedter, Axonal synapse sorting in medial entorhinal cortex. *Nature* **549**, 469–475 (2017). doi:10.1038/nature24005 Medline
7. Y. Han, J. M. Kebschull, R. A. A. Campbell, D. Cowan, F. Imhof, A. M. Zador, T. D. Mrsic-Flogel, The logic of single-cell projections from visual cortex. *Nature* **556**, 51–56 (2018). doi:10.1038/nature26159 Medline
8. Y. Mishchenko, T. Hu, J. Spacek, J. Mendenhall, K. M. Harris, D. B. Chklovskii, Ultrastructural analysis of hippocampal neuropil from the connectomics perspective. *Neuron* **67**, 1009–1020 (2010). doi:10.1016/j.neuron.2010.08.014 Medline
9. N. Kasthuri, K. J. Hayworth, D. R. Berger, R. L. Schalek, J. A. Conchello, S. Knowles-Barley, D. Lee, A. Vázquez-Reina, V. Kaynig, T. R. Jones, M. Roberts, J. L. Morgan, J. C. Tapia, H. S. Seung, W. G. Roncal, J. T. Vogelstein, R. Burns, D. L. Sussman, C. E. Priebe, H. Pfister, J. W. Lichtman, Saturated reconstruction of a volume of neocortex. *Cell* **162**, 648–661 (2015). doi:10.1016/j.cell.2015.06.054 Medline
10. C. Calì, M. Wawrzyniak, C. Becker, B. Maco, M. Cantoni, A. Jorstad, B. Nigro, F. Grillo, V. De Paola, P. Fua, G. W. Knott, The effects of aging on neuropil structure in mouse somatosensory cortex-A 3D electron microscopy analysis of layer 1. *PLOS ONE* **13**, e0198131 (2018). doi:10.1371/journal.pone.0198131 Medline
11. A. Peters, Thalamic input to the cerebral cortex. *Trends Neurosci.* **2**, 183–185 (1979). doi:10.1016/0166-2236(79)90074-2
12. T. Binzegger, R. J. Douglas, K. A. Martin, A quantitative map of the circuit of cat primary visual cortex. *J. Neurosci.* **24**, 8441–8453 (2004). doi:10.1523/JNEUROSCI.1400-04.2004 Medline
13. H. Markram, E. Muller, S. Ramaswamy, M. W. Reimann, M. Abdellah, C. A. Sanchez, A. Ailamaki, L. Alonso-Nanclares, N. Antille, S. Arsever, G. A. A. Kahou, T. K. Berger, A. Bilgili, N. Buncic, A. Chalimourda, G. Chindemi, J.-D. Courcol, F. Delalondre, V. Delattre, S. Druckmann, R. Dumusc, J. Dynes, S. Eilemann, E. Gal, M. E. Gevaert, J.-P. Ghobril, A. Gidon, J. W. Graham, A. Gupta, V. Haenel, E. Hay, T. Heinis, J. B. Hernando, M. Hines, L. Kanari, D. Keller, J. Kenyon, G. Khazen, Y. Kim, J. G. King, Z. Kisvarday, P. Kumbhar, S. Lasserre, J.-V. Le Bé, B. R. C. Magalhães, A. Merchán-Pérez, J. Meystre, B. R. Morrice, J. Muller, A. Muñoz-Céspedes, S. Muralidhar, K. Muthurasa, D. Nachbaur, T. H. Newton, M. Nolte, A. Ovcharenko, J. Palacios, L. Pastor, R. Perin, R. Ranjan, I. Riachi, J.-R. Rodríguez, J. L. Riquelme, C. Rössert, K. Sfyrakis, Y. Shi, J. C. Shillcock, G. Silberberg, R. Silva, F. Tauheed, M. Telefont, M. Toledo-Rodriguez, T. Tränkler, W. Van Geit, J. V. Díaz, R. Walker, Y. Wang, S. M. Zaninetta, J. DeFelipe, S. L. Hill, I. Segev, F. Schürmann, Reconstruction and simulation of neocortical microcircuitry. *Cell* **163**, 456–492 (2015). doi:10.1016/j.cell.2015.09.029 Medline
14. E. L. White, G. Benshalom, S. M. Hersch, Thalamocortical and other synapses involving nonspiny multipolar cells of mouse Sml cortex. *J. Comp. Neurol.* **229**, 311–320 (1984). doi:10.1002/cne.902290303 Medline
15. G. M. Shepherd, A. Stepanyants, I. Bureau, D. Chklovskii, K. Svoboda, Geometric

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and functional organization of cortical circuits. *Nat. Neurosci.* **8**, 782–790 (2005). doi:10.1038/nn1447 Medline
16. G. A. Ascoli, L. Alonso-Nanclares, S. A. Anderson, G. Barrionuevo, R. Benavides-Piccione, A. Burkhalter, G. Buzsáki, B. Cauli, J. Defelipe, A. Fairén, D. Feldmeyer, G. Fishell, Y. Fregnac, T. F. Freund, D. Gardner, E. P. Gardner, J. H. Goldberg, M. Helmstaedter, S. Hestrin, F. Karube, Z. F. Kisvárday, B. Lambolez, D. A. Lewis, O. Marin, H. Markram, A. Muñoz, A. Packer, C. C. Petersen, K. S. Rockland, J. Rossier, B. Rudy, P. Somogyi, J. F. Staiger, G. Tamas, A. M. Thomson, M. Toledo-Rodriguez, Y. Wang, D. C. West, R. Yuste; Petilla Interneuron Nomenclature Group, Petilla terminology: Nomenclature of features of GABAergic interneurons of the cerebral cortex. *Nat. Rev. Neurosci.* **9**, 557–568 (2008). doi:10.1038/nrn2402 Medline
17. Y. Kubota, F. Karube, M. Nomura, Y. Kawaguchi, The diversity of cortical inhibitory synapses. *Front. Neural Circuits* **10**, 27 (2016). doi:10.3389/fncir.2016.00027 Medline
18. M. Helmstaedter, K. L. Briggman, S. C. Turaga, V. Jain, H. S. Seung, W. Denk, Connectomic reconstruction of the inner plexiform layer in the mouse retina. *Nature* **500**, 168–174 (2013). doi:10.1038/nature12346 Medline
19. J. S. Kim, M. J. Greene, A. Zlateski, K. Lee, M. Richardson, S. C. Turaga, M. Purcaro, M. Balkam, A. Robinson, B. F. Behabadi, M. Campos, W. Denk, H. S. Seung; EyeWirers, Space-time wiring specificity supports direction selectivity in the retina. *Nature* **509**, 331–336 (2014). doi:10.1038/nature13240 Medline
20. G. Major, M. E. Larkum, J. Schiller, Active properties of neocortical pyramidal neuron dendrites. *Annu. Rev. Neurosci.* **36**, 1–24 (2013). doi:10.1146/annurev-neuro-062111-150343 Medline
21. H. Jia, Z. Varga, B. Sakmann, A. Konnerth, Linear integration of spine Ca2+ signals in layer 4 cortical neurons in vivo. *Proc. Natl. Acad. Sci. U.S.A.* **111**, 9277–9282 (2014). doi:10.1073/pnas.1408525111 Medline
22. M. Lavzin, S. Rapoport, A. Polsky, L. Garion, J. Schiller, Nonlinear dendritic processing determines angular tuning of barrel cortex neurons in vivo. *Nature* **490**, 397–401 (2012). doi:10.1038/nature11451 Medline
23. T. Branco, B. A. Clark, M. Häusser, Dendritic discrimination of temporal input sequences in cortical neurons. *Science* **329**, 1671–1675 (2010). doi:10.1126/science.1189664 Medline
24. E. Fifková, A. Van Harreveld, Long-lasting morphological changes in dendritic spines of dentate granular cells following stimulation of the entorhinal area. *J. Neurocytol.* **6**, 211–230 (1977). doi:10.1007/BF01261506 Medline
25. H. Markram, J. Lübke, M. Frotscher, B. Sakmann, Regulation of synaptic efficacy by coincidence of postsynaptic APs and EPSPs. *Science* **275**, 213–215 (1997). doi:10.1126/science.275.5297.213 Medline
26. V. Egger, D. Feldmeyer, B. Sakmann, Coincidence detection and changes of synaptic efficacy in spiny stellate neurons in rat barrel cortex. *Nat. Neurosci.* **2**, 1098–1105 (1999). doi:10.1038/16026 Medline
27. M. Matsuzaki, N. Honkura, G. C. Ellis-Davies, H. Kasai, Structural basis of long-term potentiation in single dendritic spines. *Nature* **429**, 761–766 (2004). doi:10.1038/nature02617 Medline
28. I. Sáez, M. J. Friedlander, Plasticity between neuronal pairs in layer 4 of visual cortex varies with synapse state. *J. Neurosci.* **29**, 15286–15298 (2009). doi:10.1523/JNEUROSCI.2980-09.2009 Medline
29. T. M. Bartol Jr., C. Bromer, J. Kinney, M. A. Chirillo, J. N. Bourne, K. M. Harris, T. J. Sejnowski, Nanoconnectomic upper bound on the variability of synaptic plasticity. *eLife* **4**, e10778 (2015). doi:10.7554/eLife.10778 Medline
30. K. E. Sorra, K. M. Harris, Occurrence and three-dimensional structure of multiple synapses between individual radiatum axons and their target pyramidal cells in hippocampal area CA1. *J. Neurosci.* **13**, 3736–3748 (1993). doi:10.1523/JNEUROSCI.13-09-03736.1993 Medline
31. W. Denk, H. Horstmann, Serial block-face scanning electron microscopy to reconstruct three-dimensional tissue nanostructure. *PLOS Biol.* **2**, e329 (2004). doi:10.1371/journal.pbio.0020329 Medline
32. M. Berning, K. M. Boergens, M. Helmstaedter, SegEM: Efficient Image Analysis for High-Resolution Connectomics. *Neuron* **87**, 1193–1206 (2015). doi:10.1016/j.neuron.2015.09.003 Medline
33. B. Staffler, M. Berning, K. M. Boergens, A. Gour, P. V. Smagt, M. Helmstaedter, SynEM, automated synapse detection for connectomics. *eLife* **6**, e26414 (2017). doi:10.7554/eLife.26414 Medline
34. K. M. Boergens, M. Berning, T. Bocklisch, D. Bräunlein, F. Drawitsch, J. Frohnhofen, T. Herold, P. Otto, N. Rzepka, T. Werkmeister, D. Werner, G. Wiese, H. Wissler, M. Helmstaedter, webKnossos: Efficient online 3D data annotation for connectomics. *Nat. Methods* **14**, 691–694 (2017). doi:10.1038/nmeth.4331 Medline
35. K. Eichler, F. Li, A. Litwin-Kumar, Y. Park, I. Andrade, C. M. Schneider-Mizell, T. Saumweber, A. Huser, C. Eschbach, B. Gerber, R. D. Fetter, J. W. Truman, C. E. Priebe, L. F. Abbott, A. S. Thum, M. Zlatic, A. Cardona, The complete connectome of a learning and memory centre in an insect brain. *Nature* **548**, 175–182 (2017). doi:10.1038/nature23455 Medline
36. M. Helmstaedter, K. L. Briggman, W. Denk, High-accuracy neurite reconstruction for high-throughput neuroanatomy. *Nat. Neurosci.* **14**, 1081–1088 (2011). doi:10.1038/nn.2868 Medline
37. H. Taniguchi, J. Lu, Z. J. Huang, The spatial and temporal origin of chandelier cells in mouse neocortex. *Science* **339**, 70–74 (2013). doi:10.1126/science.1227622 Medline
38. R. Bopp, S. Holler-Rickauer, K. A. Martin, G. F. Schuhknecht, An ultrastructural study of the thalamic input to layer 4 of primary motor and primary somatosensory cortex in the mouse. *J. Neurosci.* **37**, 2435–2448 (2017). doi:10.1523/JNEUROSCI.2557-16.2017 Medline
39. J. D. Storey, R. Tibshirani, Statistical significance for genomewide studies. *Proc. Natl. Acad. Sci. U.S.A.* **100**, 9440–9445 (2003). doi:10.1073/pnas.1530509100 Medline
40. M. W. Reimann, J. G. King, E. B. Muller, S. Ramaswamy, H. Markram, An algorithm to predict the connectome of neural microcircuits. *Front. Comput. Neurosci.* **9**, 120 (2015). doi:10.3389/fncom.2015.00120 Medline
41. M. Oberlaender, A. Ramirez, R. M. Bruno, Sensory experience restructures thalamocortical axons during adulthood. *Neuron* **74**, 648–655 (2012). doi:10.1016/j.neuron.2012.03.022 Medline
42. C. E. Cheetham, S. J. Barnes, G. Albieri, G. W. Knott, G. T. Finnerty, Pansynaptic enlargement at adult cortical connections strengthened by experience. *Cereb. Cortex* **24**, 521–531 (2014). doi:10.1093/cercor/bhs334 Medline
43. L. de Vivo, M. Bellesi, W. Marshall, E. A. Bushong, M. H. Ellisman, G. Tononi, C. Cirelli, Ultrastructural evidence for synaptic scaling across the wake/sleep cycle. *Science* **355**, 507–510 (2017). doi:10.1126/science.aah5982 Medline
44. E. B. Bloss, M. S. Cembrowski, B. Karsh, J. Colonell, R. D. Fetter, N. Spruston, Single excitatory axons form clustered synapses onto CA1 pyramidal cell dendrites. *Nat. Neurosci.* **21**, 353–363 (2018). doi:10.1038/s41593-018-0084-6 Medline
45. M. C. Crair, R. C. Malenka, A critical period for long-term potentiation at thalamocortical synapses. *Nature* **375**, 325–328 (1995). doi:10.1038/375325a0 Medline
46. H. Li, S. Fertuzinhos, E. Mohns, T. S. Hnasko, M. Verhage, R. Edwards, N. Sestan, M. C. Crair, Laminar and columnar development of barrel cortex relies on thalamocortical neurotransmission. *Neuron* **79**, 970–986 (2013). doi:10.1016/j.neuron.2013.06.043 Medline
47. J. Grutzendler, N. Kasthuri, W. B. Gan, Long-term dendritic spine stability in the adult cortex. *Nature* **420**, 812–816 (2002). doi:10.1038/nature01276 Medline
48. J. T. Trachtenberg, B. E. Chen, G. W. Knott, G. Feng, J. R. Sanes, E. Welker, K. Svoboda, Long-term in vivo imaging of experience-dependent synaptic plasticity in adult cortex. *Nature* **420**, 788–794 (2002). doi:10.1038/nature01273 Medline
49. D. Feldmeyer, V. Egger, J. Lubke, B. Sakmann, Reliable synaptic connections between pairs of excitatory layer 4 neurones within a single 'barrel' of developing rat somatosensory cortex. *J. Physiol.* **521**, 169–190 (1999). doi:10.1111/j.1469-7793.1999.00169.x Medline
50. C. K. Pfeffer, M. Xue, M. He, Z. J. Huang, M. Scanziani, Inhibition of inhibition in visual cortex: The logic of connections between molecularly distinct interneurons. *Nat. Neurosci.* **16**, 1068–1076 (2013). doi:10.1038/nn.3446 Medline
51. J. Yu, H. Hu, A. Agmon, K. Svoboda, Recruitment of GABAergic interneurons in the barrel cortex during active tactile behavior. *Neuron* S0896-6273(19)30653-1 (2019). doi:10.1016/j.neuron.2019.07.027 Medline
52. J. T. Porter, C. K. Johnson, A. Agmon, Diverse types of interneurons generate thalamus-evoked feedforward inhibition in the mouse barrel cortex. *J. Neurosci.* **21**, 2699–2710 (2001). doi:10.1523/JNEUROSCI.21-08-02699.2001 Medline
53. K. L. Briggman, M. Helmstaedter, W. Denk, Wiring specificity in the direction-selectivity circuit of the retina. *Nature* **471**, 183–188 (2011). doi:10.1038/nature09818 Medline

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54. M. Januszewski, J. Kornfeld, P. H. Li, A. Pope, T. Blakely, L. Lindsey, J. Maitin-Shepard, M. Tyka, W. Denk, V. Jain, High-precision automated reconstruction of neurons with flood-filling networks. *Nat. Methods* **15**, 605–610 (2018). doi:10.1038/s41592-018-0049-4 Medline
55. J. Kornfeld, S. E. Benezra, R. T. Narayanan, F. Svara, R. Egger, M. Oberlaender, W. Denk, M. A. Long, EM connectomics reveals axonal target variation in a sequence-generating network. *eLife* **6**, e24364 (2017). doi:10.7554/eLife.24364 Medline
56. D. Rolnick, N. Shavit, Morphological error detection in 3D segmentations. arXiv:1705.10882 [cs.CV] (30 May 2017).
57. J. Zung, I. Tartavull, K. Lee, H. S. Seung, An error detection and correction framework for connectomics. arXiv:1708.02599 [cs.CV] (8 August 2017).
58. Y. Hua, P. Laserstein, M. Helmstaedter, Large-volume en-bloc staining for electron microscopy-based connectomics. *Nat. Commun.* **6**, 7923 (2015). doi:10.1038/ncomms8923 Medline
59. A. A. Wanner, C. Genoud, R. W. Friedrich, 3-dimensional electron microscopic imaging of the zebrafish olfactory bulb and dense reconstruction of neurons. *Sci. Data* **3**, 160100 (2016). doi:10.1038/sdata.2016.100 Medline
60. S. Dorkenwald, P. J. Schubert, M. F. Killinger, G. Urban, S. Mikula, F. Svara, J. Kornfeld, Automated synaptic connectivity inference for volume electron microscopy. *Nat. Methods* **14**, 435–442 (2017). doi:10.1038/nmeth.4206 Medline
61. J. Friedman, T. Hastie, R. Tibshirani, Additive logistic regression: A statistical view of boosting (with discussion and a rejoinder by the authors). *Ann. Stat.* **28**, 337–407 (2000). doi:10.1214/aos/1016218223
62. J. Corney, H. Rea, D. Clark, J. Pritchard, M. Breaks, R. Macleod, Coarse filters for shape matching. *IEEE Comput. Graph. Appl.* **22**, 65–74 (2002). doi:10.1109/MCG.2002.999789
63. R. Osada, T. Funkhouser, B. Chazelle, D. Dobkin, "Matching 3D models with shape distributions" in *Proceedings of the International Conference on Shape Modeling and Applications* (IEEE, 2001), pp. 154–166.
64. L. Yi, P. Jiantao, Z. Hongbin, W. Liu, Y. Uehara, "Thickness histogram and statistical harmonics representation for 3D model retrieval" in *Proceedings of the International Symposium on 3D Data Processing, Visualization and Transmission* (IEEE, 2004), pp. 896–903.
65. J. C. Platt, "Probabilities for SV machines" in *Advances in Large Margin Classifiers*, A. J. B. Smola, P. L. Bartlett, B. Schölkopf, D. Schuurmans, Eds. (MIT Press, 2000), pp. 61–74.
66. Y. Kawaguchi, F. Karube, Y. Kubota, Dendritic branch typing and spine expression patterns in cortical nonpyramidal cells. *Cereb. Cortex* **16**, 696–711 (2006). doi:10.1093/cercor/bhj015 Medline
67. C. M. Schneider-Mizell, S. Gerhard, M. Longair, T. Kazimiers, F. Li, M. F. Zwart, A. Champion, F. M. Midgley, R. D. Fetter, S. Saalfeld, A. Cardona, Quantitative neuroanatomy for connectomics in *Drosophila*. *eLife* **5**, e12059 (2016). doi:10.7554/eLife.12059 Medline
68. B. A. McGuire, J. P. Hornung, C. D. Gilbert, T. N. Wiesel, Patterns of synaptic input to layer 4 of cat striate cortex. *J. Neurosci.* **4**, 3021–3033 (1984). doi:10.1523/JNEUROSCI.04-12-03021.1984 Medline
69. J. Lübke, V. Egger, B. Sakmann, D. Feldmeyer, Columnar organization of dendrites and axons of single and synaptically coupled excitatory spiny neurons in layer 4 of the rat barrel cortex. *J. Neurosci.* **20**, 5300–5311 (2000). doi:10.1523/JNEUROSCI.20-14-05300.2000 Medline
70. B. Ahmed, J. C. Anderson, R. J. Douglas, K. A. Martin, J. C. Nelson, Polyneuronal innervation of spiny stellate neurons in cat visual cortex. *J. Comp. Neurol.* **341**, 39–49 (1994). doi:10.1002/cne.903410105 Medline
71. D. Feldmeyer, A. Roth, B. Sakmann, Monosynaptic connections between pairs of spiny stellate cells in layer 4 and pyramidal cells in layer 5A indicate that lemniscal and paralemniscal afferent pathways converge in the infragranular somatosensory cortex. *J. Neurosci.* **25**, 3423–3431 (2005). doi:10.1523/JNEUROSCI.5227-04.2005 Medline
72. H. S. Meyer, V. C. Wimmer, M. Oberlaender, C. P. J. de Kock, B. Sakmann, M. Helmstaedter, Number and laminar distribution of neurons in a thalamocortical projection column of rat vibrissal cortex. *Cereb. Cortex* **20**, 2277–2286 (2010). doi:10.1093/cercor/bhq067 Medline

ACKNOWLEDGMENTS

We thank Y. Hua and K. Harris for discussions; A. Gour, Y. Hua, P. Laserstein, and H. Schmidt for discussing unpublished observations; B. Cowgill for experimental contributions; E. Eulig, R. Hesse, C. Schramm, and M. Zecevic for code contributions; C. Guggenberger for compute infrastructure, scalable minds for implementation of the fast-swapping user queries in webKnossos, and annotators (see supplementary materials) for reconstruction work. **Funding:** Funding was provided by the Max Planck Society. **Author contributions**: M.H. conceived, initiated, and supervised the project; K.M.B. performed experiments; P.H. provided analysis methods; A.M., M.B., K.M.B., and B.S. developed and performed analyses with contributions by all authors; A.M. and M.H. wrote the manuscript with contributions by all authors. **Competing interests**: The authors declare no competing financial interests. **Data and materials availability**: The raw EM data are available for browsing via https://wklink.org/9276 (see data links in figure legends). All data and code will be made permanently publicly available at https://l4dense2019.brain.mpg.de and in updated form at https://gitlab.mpcdf.mpg.de/connectomics/l4dense upon publication. Less than 2% of the 3D EM image dataset using the initial image alignment was previously published (*32*–*34*, *58*).

SUPPLEMENTARY MATERIALS

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Materials and Methods
Supplementary Text
Figs. S1 to S7
Tables S1 and S2
Captions for Movies S1 and S2
References (*60*–*72*)

7 June 2019; accepted 11 October 2019
Published online 24 October 2019
10.1126/science.aay3134

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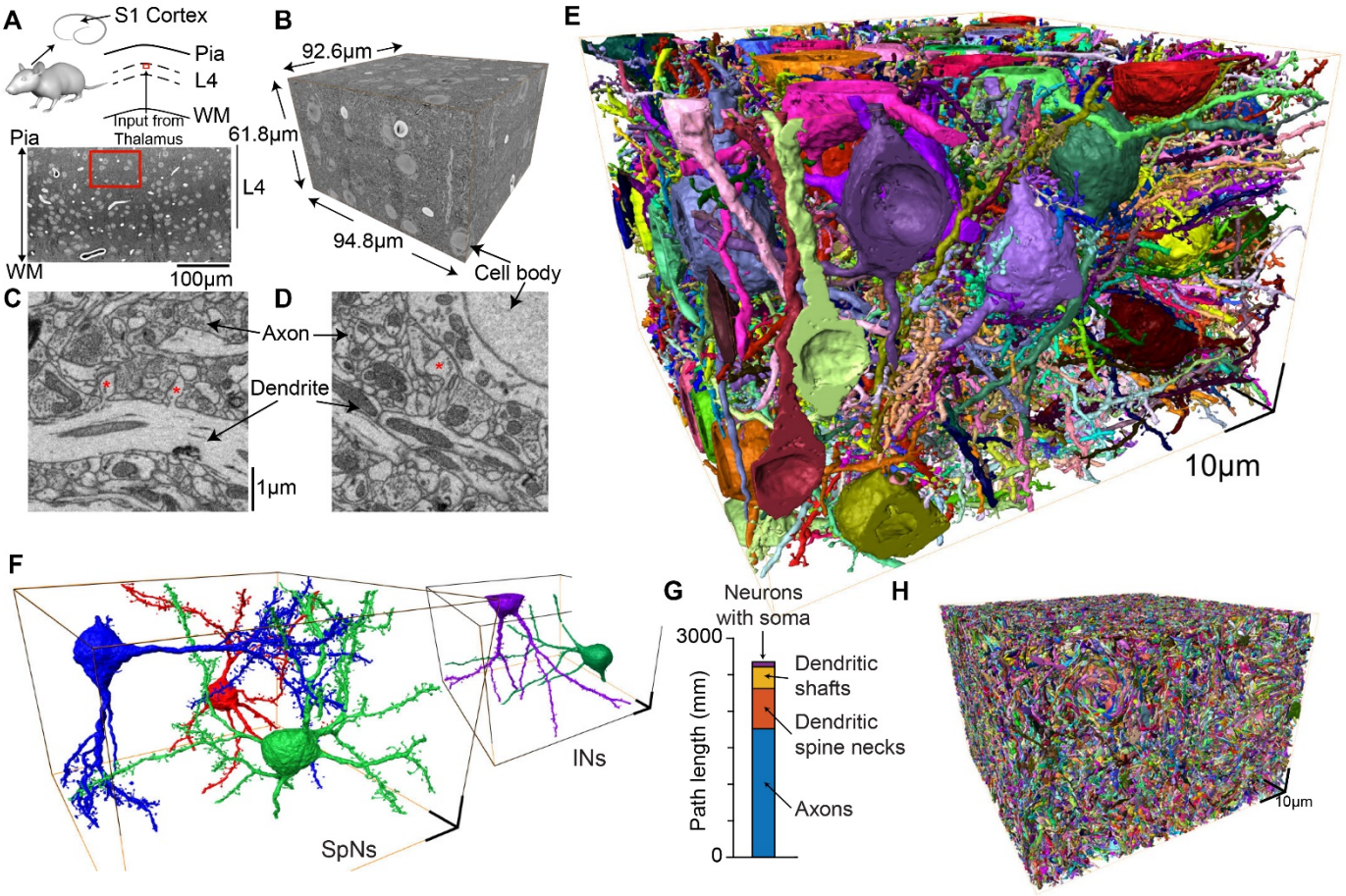

Fig. 1. Dense connectomic reconstruction of cortical neuropil from layer 4 of mouse primary somatosensory cortex. (**A** to **D**) Location (A, red) of 3D EM dataset (B), WM: white matter; high-resolution example images (**C** and **D**). Asterisk, examples of dendritic spines. Direct links to data browser webknossos: https://wklink.org/9276 (B), https://wklink.org/7101 (C), https://wklink.org/8906 (D). (**E**) Reconstruction of all n=89 neurons with a cell body and dendrites in the dataset. (**F**) 3 spiny neurons (SpNs) and 2 interneurons (INs); see movie S1. (**G**) Quantification of circuit components in the dense reconstruction. Note the majority of circuit path length (total: 2.69 m) is contributed by non-proximal axons (1.79 m, 66.6%), spine necks (0.55 m, 20.5%), and dendritic shafts (0.28 m, 10.3%) not connected to any cell body in the volume. (**H**) Display of all reconstructed 34,221 axons contained in the dataset. Scale bars (D) as (C); 10 µm (F).

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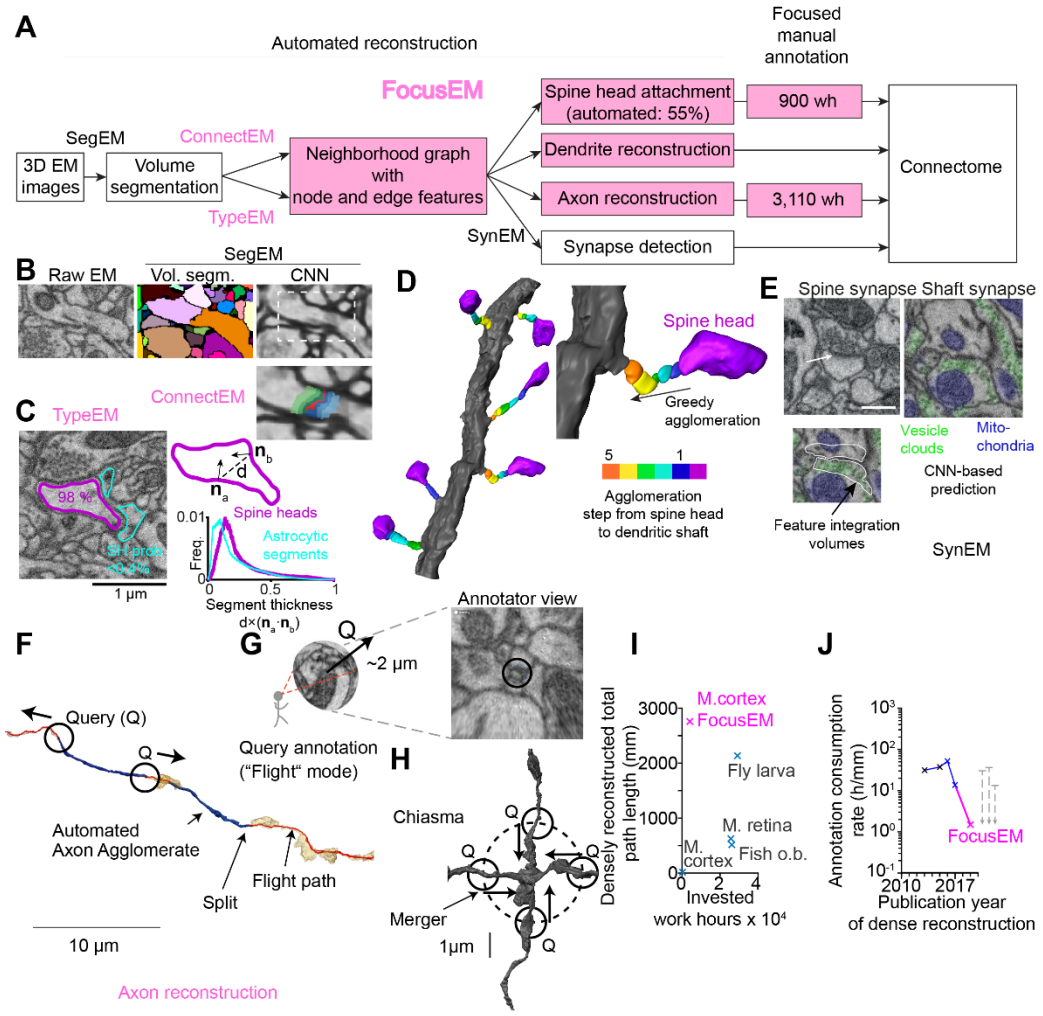

Fig. 2. Methods for the efficient dense connectomic reconstruction. (**A**) Simplified diagram of reconstruction steps [fig. S1, detailed in (B) to (H)]; wh: annotation work hours. (**B**) ConnectEM-Classifier for combining neurite pieces from the CNN-based volume segmentation (*32*): at junctions of volume segments (bottom right), raw data, CNN and shape features were evaluated. (**C**) TypeEM: classifier for assigning cellular identity to volume segments: probability of axons, dendrites, spine heads and glial processes. Illustration of spine head (purple) and astrocyte (cyan) classification; one of the 985 features is illustrated (segment thickness). Numbers: probability of the segment to be a spine head. Precision and recall of spine head detection were 92.6 and 94.4%, respectively. (**D**) Process for automatically attaching spine heads to dendritic shaft by stepwise agglomeration of volume segments along the highest-probability transition between neighboring segments [according to the ConnectEM score (B)]. Example of 6 neighboring spine heads that were all automatically attached. In total, 58.9% of spine heads were automatically attached (A). (**E**) Automated detection of spine and shaft synapses (here, vesicle clouds (green) and mitochondria (blue) were classified and used as additional features for the SynEM (*33*) classifier). (**F** to **H**) Focused annotation strategy for directing human annotation queries (Q, red) to ending locations of the automatically reconstructed axon pieces (F, blue), oriented along the axon's main axis (traced in webKnossos using flight mode [G (*34*)] yielding flight paths of 5.5 ± 8.8 µm length (21.3 ± 36.1s per annotation, n=242,271, movie S2). Neurite mergers (H) were detected as "chiasmatic" configurations, and queries (Q) directed from the exits of the chiasma toward its center to determine correct neurite continuities (fig. S1). (**I** and **J**) Quantification of circuit size and invested work hours for dense circuit reconstructions in connectomics, and resulting order-of-magnitude improvement provided by FocusEM compared to previous dense reconstructions (m). Fish o.b.: Zebrafish olfactory bulb (*59*); M. retina: Mouse retina IPL (*18*); Fly larva: mushroom body in larval stage of D. melanogaster (*35*); M. cortex: (*9*) and this study (magenta). Only completed dense reconstructions were included in the comparison.

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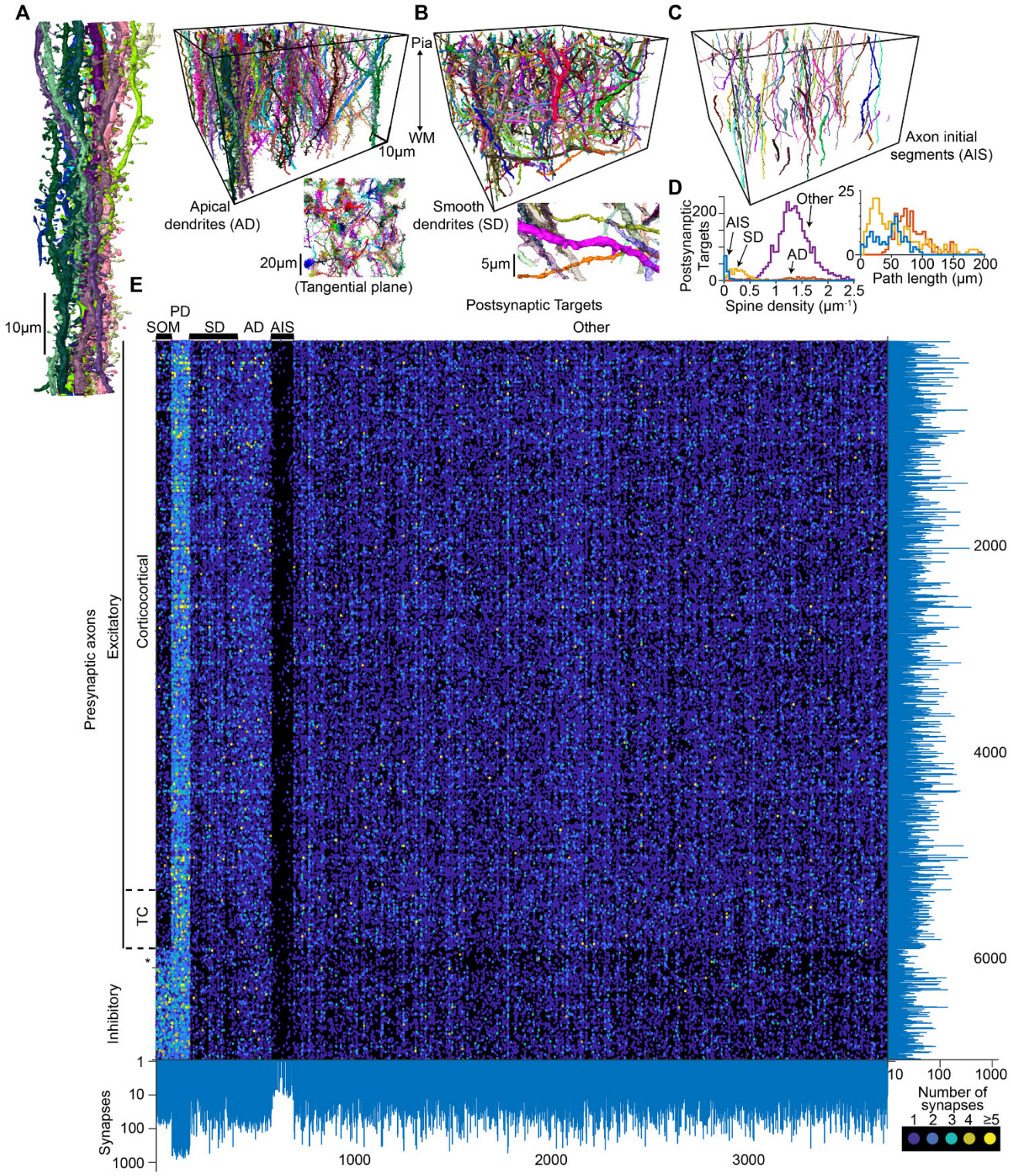

Fig. 3. Postsynaptic target classes and dense cortical connectome. (**A** to **D**) Display of all apical dendrites (AD, A, magnified one apical dendrite bundle (left), and top view in tangential plane illustrating AD bundles), smooth dendrites (SD) [(B), magnification inset illustrates low rate of spines], axon initial segments (AIS) (C) and their respective path length and spine density distributions (D). Note spine density is underestimated by about 20% (table S1). (**E**) Display of connectome between all axons (n=6,979) and postsynaptic targets (n=3,719) in the volume with at least 10 synapses, each; total of 153,171 synapses (of 388,554 synapses detected in the volume). For definition of postsynaptic target classes, see (A) to (D); definition of presynaptic axon classes: see Fig. 4 and fig. S6. AIS with less than 10 input synapses shown. SOM: neuronal somata; PD: proximal dendrites connected to a soma in the dataset (note that some of these PD dendrites are L4 apical dendrites not included in the AD definition above); asterisk, remaining unassigned axons.

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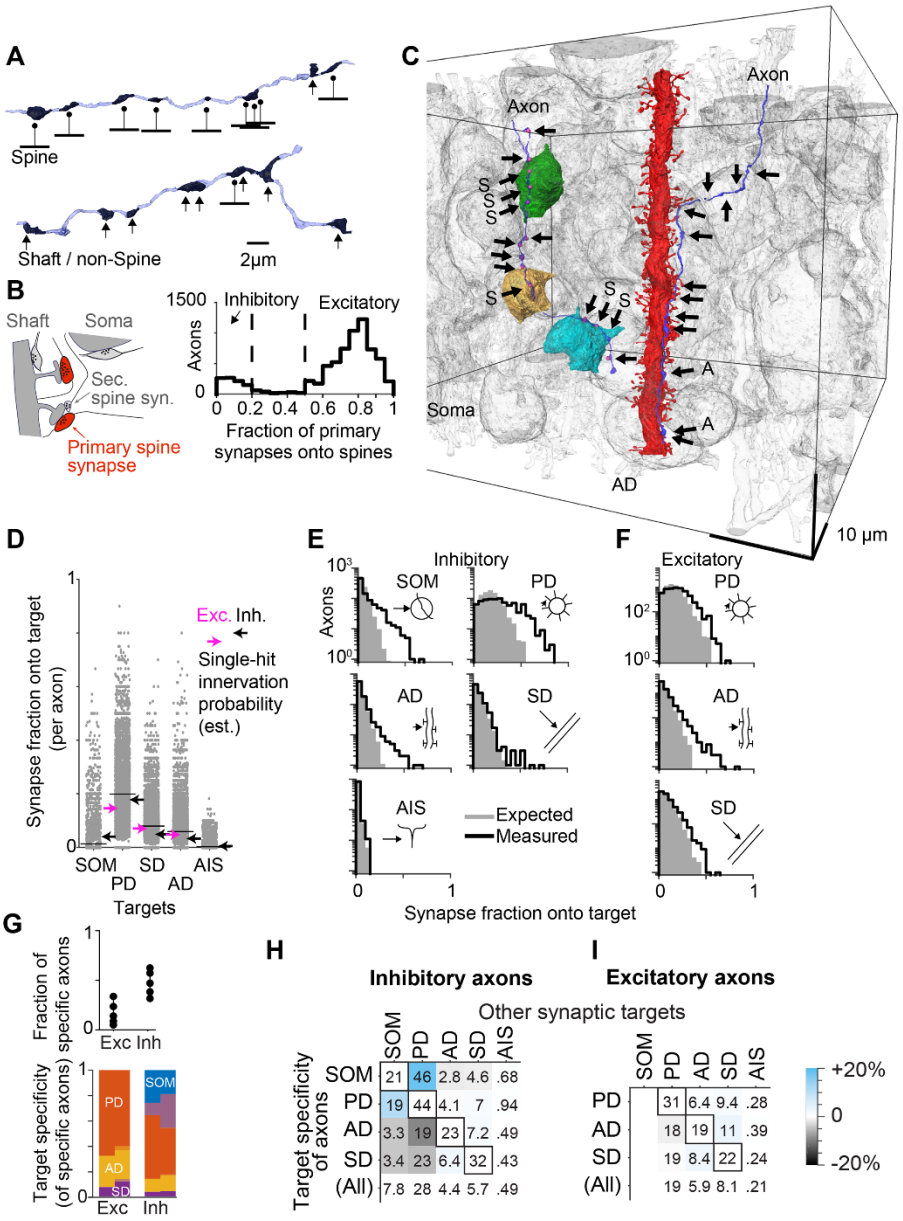

Fig. 4. Connectomic definition of axon classes. (**A**) Example axons with high (top) and low (bottom) fraction of output synapses made onto dendritic spines. (**B**) Distribution of spine targeting fraction over all n=6,979 axons; dashed lines indicates thresholds applied to distinguish non-spine preferring, likely inhibitory (<20% spine innervation, n=893, 12.8% of all axons) from spine-preferring, mostly excitatory (>50% spine innervation, n=5,894, 84.5%) axons. Sketch: definition of primary spine innervations. (**C** to **I**) Connectomic definition of axon classes via preferential synaptic innervation of subcellular targets. (C) Two example axons innervating three somata (left, n=6 synapses onto somata (S) of 14 total, arrows) and an apical dendrite (AD, right, n=2 synapses onto AD of 13 total), respectively. All other cell bodies and ADs in gray. (D) Fraction of synapses onto Somata, PDs, ADs, SDs, AIS for all axons. Binomial probabilities over axons to establish at least one synapse onto the respective target (arrows; magenta, excitatory; black, inhibitory). Black lines, average over axons. (E) Comparison of predicted synapse fraction onto target classes per inhibitory axon based on the binomial probability to innervate the target at least once (gray shaded, see arrows in D) and measured distribution of synapse fractions onto targets (black lines). (F) Same as (E) for excitatory axons. (G) Fraction of target-preferring excitatory (Exc) and inhibitory (Inh) axons identified using the false detection rate criterion [q=5..30% (*39*)]. Colored bars: distribution for q=5% (left) and q=30% (right). Mixed colors: axons specific for both Somata and PD. (H and I) 2nd order innervation preference by target-preferring axons; numbers report fractional innervation by remaining synapses per axon; colors indicate under- (black) or over-(blue)-frequent innervation. Diagonal entries: fraction of synapses onto same target (black boxes).

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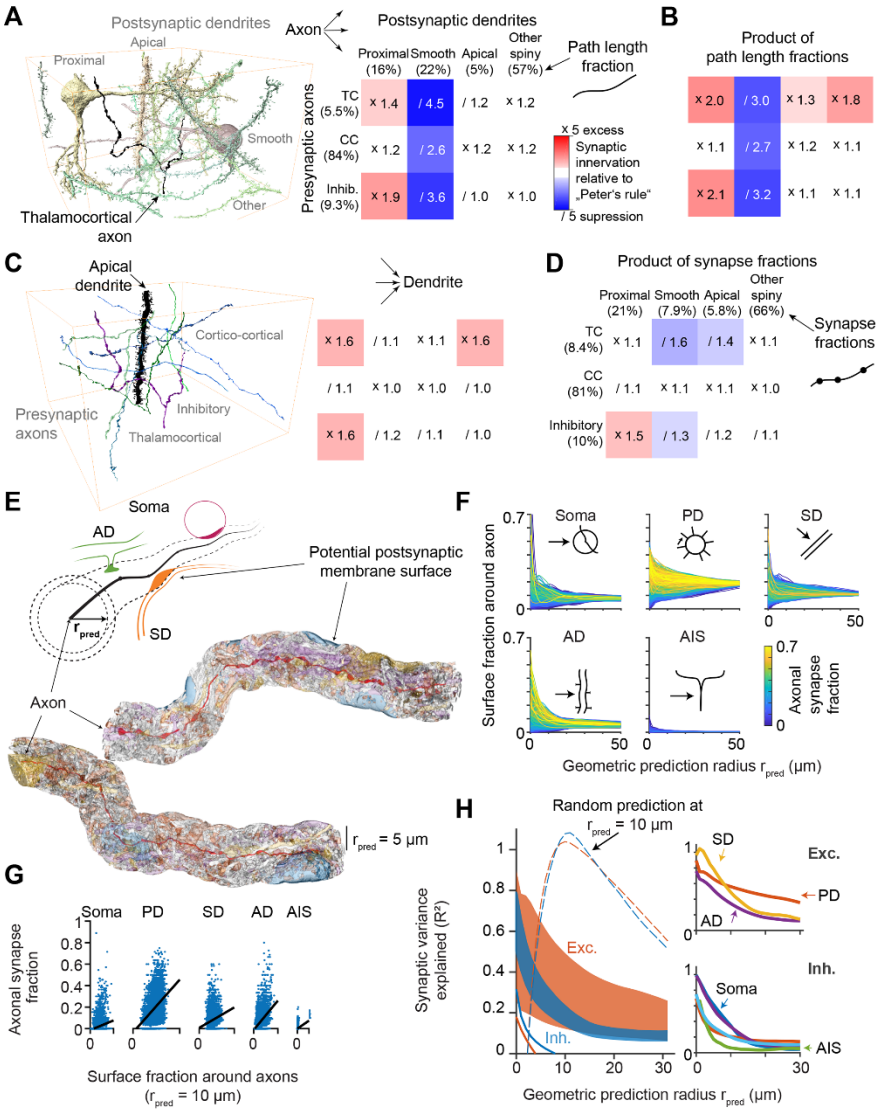

Fig. 5. Contribution of neurite geometry and membrane availability to cortical wiring. (**A** to **D**) quantitative test of various formulations of Peters' rule: comparison of actual synaptic innervation to the prediction of synaptic innervation based on the availability of postsynaptic path length in the dataset (A), the product of pre- and postsynaptic path length (B), the sampling of presynaptic partners by their relative prevalence (C) and the product of pre- and postsynaptic synapse density (D). Log likelihood ratios were: -1.1×10^3 (A), -11×10^3 (C), -12×10^3 (D), all compared to simple model in (B), p<10^{-14} (corrected for degrees of freedom). (**E** to **H**) Prediction of single-axon synaptic target preference by distance-dependent postsynaptic surface sampling. (E) Sketch of the surface area of the various subcellular postsynaptic target classes (colors) within a distance r_{pred} from a given axon (black) and example surfaces around two axons within a prediction radius r_{pred}=5µm. (F) Surface fraction of target classes around all n=6,979 axons in dependence of r_{pred} around axons. Colors: fraction of synapses of a given axon actually innervating the respective target. (G) Relation between the surface fraction around all axons and synaptic innervation by these axons for each target (r_{pred}=10µm). Black lines: linear regression for geometrical innervation prediction. (H) Coefficient of determination (R^2) reporting the fraction of synaptic innervation variance [over all axons, see (G)] explained by a multivariate linear innervation model using the available postsynaptic surface area around axons (shaded area; red, excitatory axons; blue, inhibitory axons; lower end of shades indicates prediction; upper ends indicate correction by the variance contributed by the multinomial sampling of targets along axons; solid lines represent direct prediction of innervation from surface fraction. Dashed lines: modeled prediction for a purely geometric forward model at r_{pred}=10µm. Insets (right) show sampling-corrected predictive power of excitatory (top) and inhibitory (bottom) axons for the innervation of target classes.

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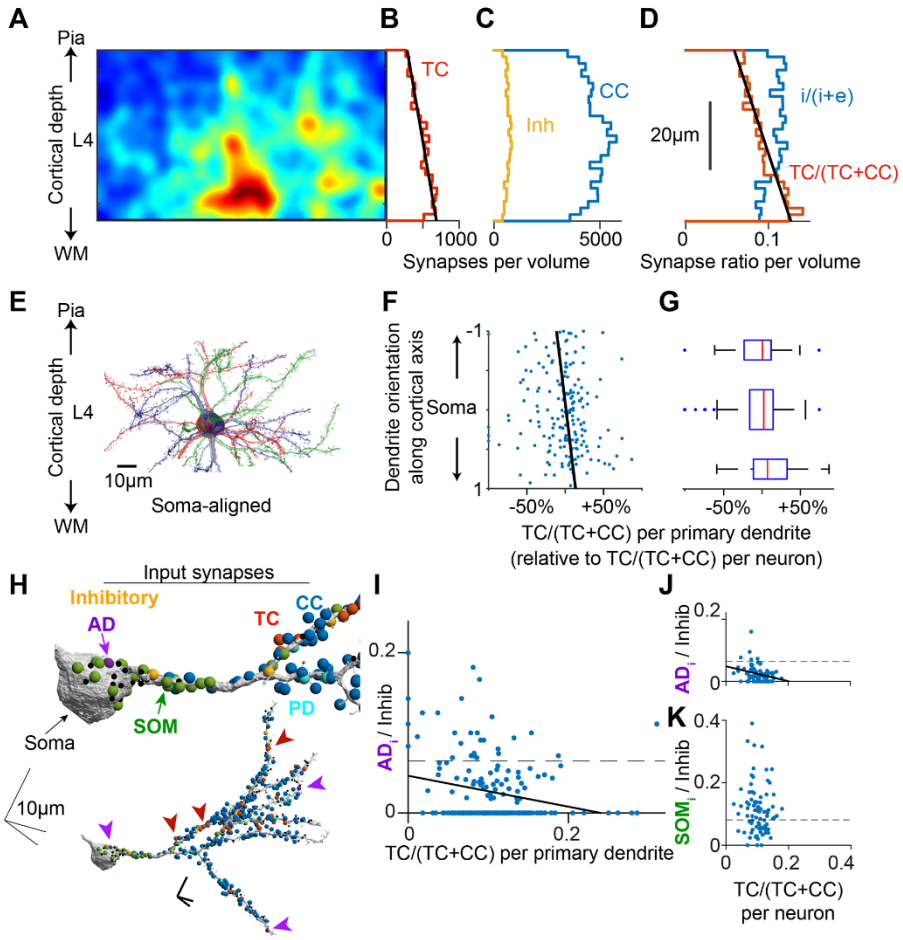

Fig. 6. Gradient of thalamocortical synapse density in L4 and ensuing variability of synaptic input composition in L4 neurons. (**A** to **D**) Distribution of TC synapses within L4 dataset (A): gradient along cortical axis (B), which is absent for inhibitory (yellow) or CC (blue) synapses (C). (D) Resulting gradient in TC synapse fraction (increase by 83% from 7.0% to 12.8% (+5.8%) within 50 µm along the cortical axis; line fit, $p<1.1\times10^{-12}$, n=134,537 synapses). (**E** to **G**) Analysis of the variability of TC input onto the primary dendrites of neurons possibly resulting from the TC synapse gradient (D): example reconstructions (E) aligned to the somata; (F) fraction of excitatory input synapses originating from TC axons evaluated for each primary dendrite, plotted according to the direction of the dendrite relative to cortical axis (-1: aligned toward pia; +1: toward WM). TC input fraction (TC/(TC+CC)) of each dendrite compared to the TC input fraction of its entire parent neuron, ratios shown. (G) Summary analysis of relation between dendrite direction and relative TC input fraction showing that TC input fraction is determined by the dendrites' orientation relative to the cortex axis (1.28-fold higher relative TC fraction for downwards than upwards pointing dendrites, n=183, p=0.026, two-sided *t* test for dendrites with a normalized absolute projection >0.5; bars correspond to ranges -1..-0.5; -0.5..0.5; 0.5..1). (**H** to **K**) Enhanced thalamocortical synaptic input (red spheres) is correlated to reduced inhibitory input from AD-preferring inhibitory axons (purple spheres and arrows in H) at the level of single dendrites (r=-0.24, p=0.0095, n=183, Pearson correlation after Bonferroni correction) and for neurons (J, r=-0.27, p=0.01, n=84), but not soma-preferring inhibitory axons (green in H, K, r=0.08, p=0.49, n=84).

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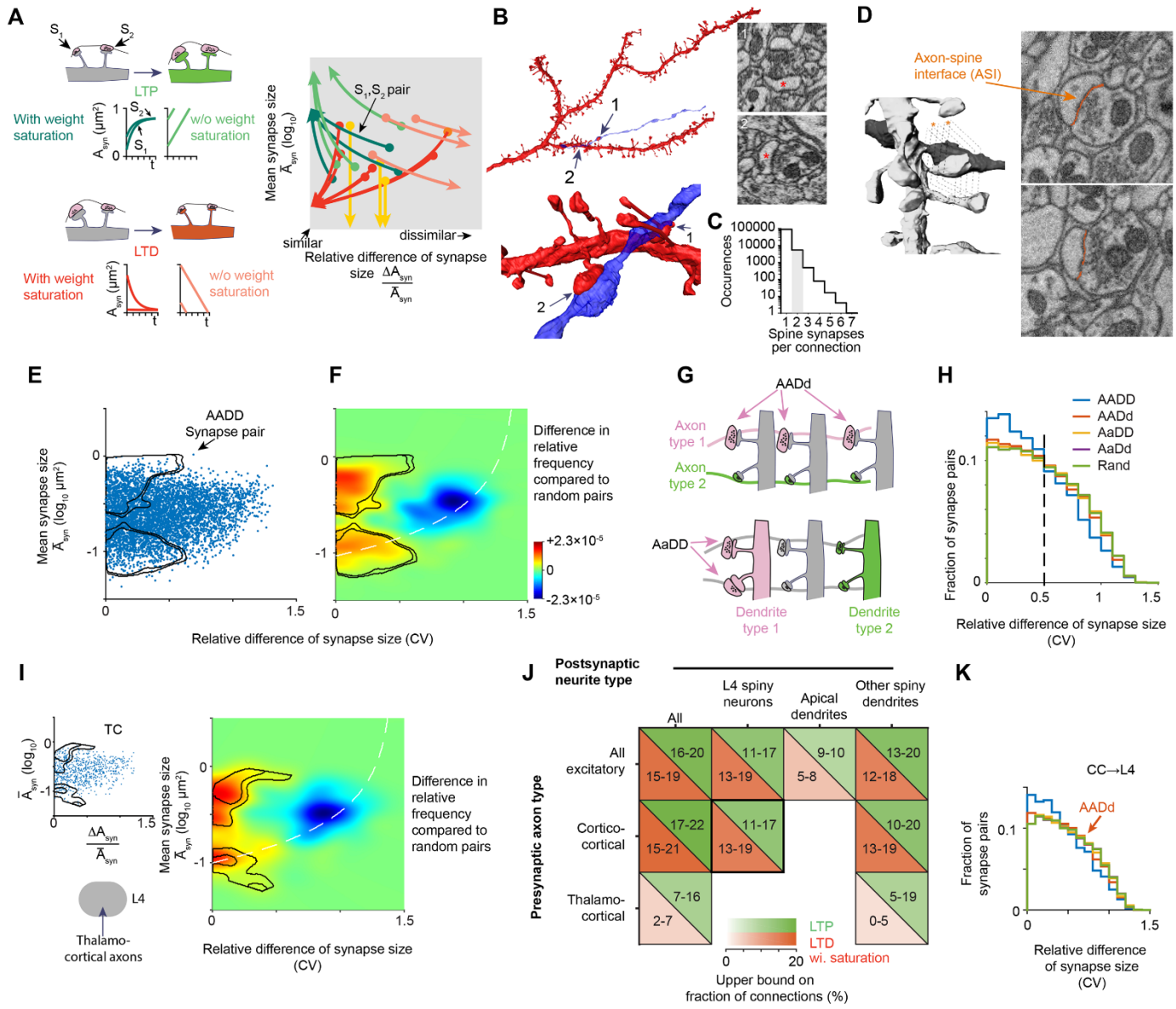

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Fig. 7. Connectomic mapping of the plasticity-consistent circuit fraction. (**A**) Hebbian LTP makes predictions about the temporal evolution of synaptic size and size similarity in pairs of synapses between the same axon and the same dendrite (green, insets show example model trajectories of synapse pairs exposed to LTP with and without weight saturation), yielding a region in the size-similarity plane (right) where synaptic pairs that have undergone LTP are predicted to be found (colors in right panel as in temporal plots on the left). For Hebbian LTD, pairs of synapses behave accordingly only if synaptic size saturates at low values (red). Arrows: trajectories of synapse pairs with randomly drawn initial size that undergo LTP with (dark green) or without (light green) weight saturation; LTD with (red) and without (rosé and yellow; linear and exponential decay, respectively) weight saturation. (**B**) Example synapse pair (arrows) onto dendritic spines between the same axon (blue) and same dendrite (red). Direct link to dataset: https://wklink.org/3356 (synapse 1), https://wklink.org/6145 (synapse 2). (**C**) Frequency of joint synapse pairs in the dataset (n=5,290 spine-synapse pairs, shaded, analyzed here). (**D**) Axon-spine-interface (ASI) as representative measure of synapse weight (*42*, *43*). https://wklink.org/5780 (**E**) Distribution of mean synaptic size and synaptic size similarity (coefficient of variation, CV) for all pairs of synapses between same excitatory axon and same dendrite (AADD, connections onto spines, only), each dot corresponds to one synapse pair. Isolines: statistical regions defined in (F). (**F**) Map of the relation between synaptic size and synaptic size similarity in same-axon same-dendrite synapse pairs, reported as difference of (E) to random synapse pairs (fig. S7, C and D). Isolines: significance levels (p=0.05;0.005) outlining over-frequency of synapse pairs that are similar in size and large (upper area), and similar and small (lower area). (**G** and **H**) Analysis of same-axon different-dendrite and same-dendrite different-axon synapse pairs which would indicate a contribution of cell-type dependent connection size differences. No over-similarity can be found in these cases (H). (**I**) Analysis as in (E) and (F) for thalamocortical connections (TC). Note upper bound of 15% of connections consistent with stabilized LTP. (**J** and **K**) Summary of fraction of synapse pairs that resided in the regions identified in (F and I) as significantly over-abundant as upper bounds, for interaction between the two upper bounds, see supplementary materials). Numbers: ranges for different significance thresholds [see (F) and (I) and fig. S7, C and D). (K) Analysis as in (G) and (H) for CC-to-L4 neuron connections, only, refuting subtypes of CC connections as the source of the observed oversimilarity (see fig. S7, A and B). 2 µm image width (B and D).

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Dense connectomic reconstruction in layer 4 of the somatosensory cortex

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published online October 24, 2019

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