Clustered thalamocortical input onto layer 5 pyramidal neurons detected using quantitative large-scale array tomography

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Abstract

The subcellular locations of synapses on pyramidal neurons strongly influences dendritic integration and synaptic plasticity. Despite this, there is little quantitative data on spatial distributions of specific types of synaptic input. Here we use array tomography (AT), a high-resolution optical microscopy method, to examine thalamocortical (TC) input onto layer 5 pyramidal neurons. We verify the ability of AT to identify synapses by means of parallel electron microscopic analysis. We then use large-scale AT to measure TC synapse distribution in a 1.00 x 0.83 x 0.21 mm^3 volume of mouse somatosensory cortex. We found that TC synapses primarily target basal dendrites in layer 5, but also make a considerable input to proximal apical dendrites in L4, consistent with previous work. However, we also found that TC inputs are biased towards certain branches and, within branches, synapses show significant clustering with an excess of TC synapse nearest neighbors within 5-15 μm compared to a random distribution. Thus, we show that AT is a sensitive and quantitative method to map specific types of synaptic input on the dendrites of entire neurons. We anticipate that this technique will be of wide utility for mapping functionally-relevant anatomical connectivity in neural circuits.

Keywords

array tomography; electron microscopy; thalamocortical synapse; dendritic integration; synapse distribution; barrel cortex; neural circuits

Introduction

Cortical pyramidal neurons receive input via thousands of synapses distributed over the entire dendritic tree. The functional efficacy of synapses is influenced by their electrotonic distance from the soma, the presence of local active conductances, and biochemical compartmentalization and proximity to co-active synapses at the μm scale (Benshalom and White, 1986; Polsky et al., 2004; Peters and Payne, 1993; London and Hausser, 2005; Ahmed et al., 1994; Yasuda et al., 2006; Douglas et al., 1995; Stratford et al., 1996; Alonso et al., 1996; Gil et al., 1999; Beierlein et al., 2002; Bruno and Sakmann, 2006; Harvey and Svoboda, 2007; Harvey et al., 2008). The subcellular location of synapses is therefore a critical factor in shaping their functional impact. On thalamo-recipient dendrite in primary sensory cortex, thalamocortical input accounts for only a small fraction of the total synapses (Polsky et al., 2004; Benshalom and White, 1986; London and Hausser, 2005; Peters and Payne, 1993; Yasuda et al., 2006; Ahmed et al., 1994; Douglas et al., 1995; Stratford et al., 1996; Alonso et al., 1996; Gil et al., 1999; Beierlein et al., 2002; Bruno and Sakmann, 2006), yet, functionally, the input is thought to be one of the strongest. The explanation for this anatomical-functional disparity is unclear. One prominent yet largely untested hypothesis (Larkum and Nevian, 2008) is that the dendritic location and clustering of the input could be an important factor in determining its strength.

The current lack of knowledge about the subcellular distribution of synapses is largely due to a lack of appropriate techniques. Calcium imaging can be used to detect active synapses; however, this approach lacks single synapse resolution and large scale capacity (Petreanu et al., 2009; Richardson et al., 2009; Bagnall et al., 2011). Optogenetic approaches lack fine-scale resolution (Petreanu et al., 2009) but see (Little and Carter, 2012). Another approach uses over expressed GFP-fusions of interacting pre- and postsynaptic proteins such that fluorescence occurs only when they in very close proximity (‘GRASP’)(Feinberg et al., 2008; Kim et al., 2012). Although promising, this approach requires overexpression of synaptic proteins that may affect synaptic and network function (Scheiffele et al., 2000; Graf et al., 2004). Furthermore, GRASP relies

on molecular targeting to synapses and thus may have to be redeveloped anew for different types of neurons.

The ideal technique requires the necessary sensitivity to reliably detect individual synapses and to resolve individual synapses. The method should be applicable to a sufficiently large volume of tissue to allow reconstruction of entire dendritic trees and needs to be combined with labeling of specific input. Synapses are easily resolved using electron microscopy (EM); however, this method is rarely used for reconstruction of very large volumes, and is not easily compatible with labeling methods while preserving ultrastructure. While conventional fluorescence light microscopy allows large scale imaging and is compatible with labeling of specific synaptic inputs, its resolution is insufficient for resolving synapses in tissue (Mishchenko, 2010).

We reasoned that array tomography (AT) has many of the critical attributes. AT is a high-resolution, wide-field fluorescence imaging technique based on repeated imaging of arrays of ultrathin serial sections, followed by computational reconstruction into a three-dimensional volume (Micheva and Smith, 2007; Micheva et al., 2010). The use of ultrathin sections enables isotropic resolution and reliable and repeatable immunostaining of synaptic proteins. Moreover, this technique can be combined with the use of molecular-genetic approaches to express fluorescent reporters in specified neuronal populations enabling labeling of synaptic inputs of specific origin. Finally, sufficiently large volumes can be imaged, potentially encompassing entire dendritic trees. It has not yet been determined if AT can reliably resolve individual synapses.

Here we characterize large-scale AT (LSAT) for mapping specific types of inputs within the dendritic arbors of individual neurons. We focus on TC input onto the dendritic trees of layer (L) 5 pyramidal neurons. We compare use AT and EM 3D stacks to quantify the accuracy and reliability of AT synapse detection. We then perform LSAT (1.00 x 0.83 x 0.21 mm^3 volume) on a block of mouse somatosensory cortex and locate TC synapses on the dendritic trees of a number of L5 pyramidal neurons. This data set suggests that TC

inputs exhibits weak clustering and dendritic branch preference, and demonstrates the power and utility of this approach.

Results

To study the subcellular distribution of TC synapses onto L5 pyramidal neurons in primary somatosensory barrel cortex with LSAT, we labeled pre- and postsynaptic groups of neurons with different fluorescent proteins (Figure 1A). Postsynaptic neurons were labeled in Thy1-YFP (type H) mice in which cortical L5 neurons sparsely express YFP (Feng et al., 2000). We labeled neurons in the ventral posteriomedial nucleus of thalamus (VPm), which project to barrel cortex, with adeno-associated virus (AAV) expressing tdTomato. Adult mice were stereotaxically injected with AAV. After ~4 weeks of expression the brain was fixed by transcardial perfusion. The primary somatosensory cortex was embedded in acrylic resin. Serial ultrathin sections (200 nm) were made from the embedded block covering a volume of ~0.2 mm^3. Each section was stained with an anti-synaptophysin antibody to label presynaptic terminals, and DsRed and GFP antibodies to enhance the signal from the encoded expressed pre- and postsynaptic fluorophores (Figure 1B, C). Sections were then imaged and reconstructed in three dimensions (Figure 1D2). In a separate experiment we quantified the accuracy and reliability of synapse identification by AT. For this work we focused on L4 because of the higher density of TC synapses. L4 neurons were labeled with GFP using an AAV expressing FLEX-reverse GFP (Atasoy et al., 2008) injected into primary somatosensory cortex of six3-CRE mice (which express CRE recombinase in L4, but not other neocortical layers, (Liao and Xu, 2008)). AT fluorescence microscopy and electron microscopy were performed on the same serial sections (Figure 1D1).

Large Scale Array Tomography (LSAT) of TC synapses on L5 pyramidal neurons

For LSAT we prepared 1074 serial ribbon sections (thickness, 200 nm) of barrel cortex and immunostained each as above. Imaging was carried out on an automated Zeiss Observer inverted fluorescence microscope using a 100x, 1.45 N.A.objective and custom autofocussing (see Experimental procedures). Each section (area, 1.00 x 0.83 mm^2) was imaged as a series of 240 overlapping tiles for a total of 257,760 images. The imaging time per tile was 5.3 seconds for a total of ~877.5 hours. The images were aligned in three dimensions using custom software and rendered in three-dimensions. The imaged

volume contained the somata of ~56 labeled pyramidal neurons (Figure 2A-D; Supplementary movie). We chose eight neurons for manual reconstruction because large parts of their dendritic arbors were contained in the imaged volume (Figure 2E). In some neurons the apical tufts were severed. However, TC inputs from the VPm innervate L1 and L2 only sparsely(Jensen and Killackey, 1987; Zhang and Deschenes, 1998; Petreanu et al., 2009; Oberlaender et al., 2011a; Meyer et al., 2010), suggesting that only a small fraction of TC input was missed. We also observed some enlargements of the thalamocortical axons, which may be result of overexpression of tdTomato or due to aging (Feldman and Peters, 1998). We identified putative TC synapses on these eight pyramidal neurons by manually inspecting the three-color image stacks. We scored YFP-labeled spines (green) touching tdTomato positive TC terminals (red) that were stained for synaptophysin (blue; Figure 3; see Methods). We reconstructed 4.7 ± 0.8 mm (mean ± standard deviation) of dendrites and counted 328.0 ± 31.2 TC synapses per neuron (Figure 4).

Accuracy of synapse assignment

A critical unresolved question concerns the accuracy and reliability of AT in assigning synapses to the correct dendrite because of the high density of synapses in the cortical neuropil (~1/μm^3). Although theoretical work predicts that AT can detect synapses with a high degree of accuracy (Mishchenko, 2010), this has not been rigorously tested experimentally. Thus, it is currently unknown whether AT is a truly quantitative method for synapses detection in large volume reconstruction. To address this issue, we directly compared synapse detection by AT with detection using EM on the same sections. We performed this experiment on TC inputs to L4 neurons in barrel cortex because of the higher TC synapse density in this region. Traditional EM preparation techniques quench intrinsic fluorescence of GFP and greatly reduce antigenicity. Therefore we explored the parameter space for conditions that can sufficiently preserve intrinsic fluorescence and antigenicity to allow light imaging for AT while still retaining sufficient structure in EM to reliably detect synapses. We found that a low concentration of osmium (0.001%) provided enough structural preservation for transmission EM while preserving synaptophysin antigenicity and GFP fluorescence. We compared the detection of

synaptophysin punctae in this 'correlative' EM condition to L4 barrel cortex sections prepared using a more traditional EM protocol (Figure 5). There was no difference in the number of synaptophysin punctae between these conditions. We also compared the number of postsynaptic densities (PSDs) detected in the same sections using EM. Although the ability to image membranes was diminished under the correlative EM conditions, PSDs were reliably detected and we consistently found no difference in the number of PSDs detected in L4 barrel cortex compared to traditional EM processing. Therefore, our correlative EM conditions allowed us to directly quantify the number of synapses detected both by AT and EM on the same ultrathin sections.

To directly compare synapse detection by AT and EM, we acquired light and electron microscopic images from the same serial sections (Figure 6). Since mitochondria are easily identified in EM and are GFP fluorescence-negative in light microscopy, we aligned the light and EM images using the mitochondria as unambiguous and abundant landmarks (Figure 6A-C). TC synapses were then manually assigned using the light microscopy images. Once completed, the EM images of the same stacks of sections were then examined (blind to the light microscopy synapse assignment) and TC synapses assigned using this imaging modality. The combined light and EM image stacks were then examined together and TC synapses were classified into three categories using EM image as ground truth (Figure 6C-D): (1) 'True', a presynaptic terminal containing red (tdTomato expressed in TC axon terminal) and blue (synaptophysin staining) apposed to a green spine (postsynaptic GFP expression) exhibiting a PSD; (2) 'False positive', presynaptic red and blue, postsynaptic green, but no detectable PSD apposed to presynaptic terminal; (note that these are sub-classified into two types based on whether a PSD is detected on the spine; Figure 6D), and (3) 'False negative', a TC presynaptic terminal (tdTomato and synaptophysin positive detected with light microscopy) apposed to a PSD detected by EM, but this synapse is not detected in light imaging. Using this approach, we analyzed 322 putative TC synapses and found a false positive rate of 22 ± 8.0% and a false negative rate of 14.2 ± 3.1% (Figure 6E). Therefore under our conditions, using AT alone at least 78% of TC synapses are correctly identified, and 14% are not detected.

We next compared the experimentally-determined accuracy to that obtained from a simulation of our staining and imaging conditions. Fluorescence images were simulated at varying light imaging resolutions using a data set of segmented EM images as previously described (Mishchenko et al., 2010). We then calculated the number of synaptophysin punctae observed to be apposed to each individual spine head at the different light imaging resolutions and used this to calculate a false positive rate for synapse detection by light microscopy (Figure 7A, B; see Methods for further details). The modeling predicted ~30% false positive rate at our imaging resolution (200 nm)(Figure 7C), similar to the ~22% false positive rate observed experimentally.

The distribution of TC synapses on L5 pyramidal neurons

Having established the accuracy of AT in detecting synapses, we next analyzed the distribution of the TC input to L5 pyramidal neurons in our LSAT data set. We first looked at the distribution of TC synapses relative to the soma and cortical layers. Although the dendritic trees of L5 pyramidal neurons transverse L5-L1, we found that most of the TC synapses onto this cell type are located close to the soma (Figure 8A-C; 77.9 ± 9.4% of all TC synapses within a 200 μm path length from the soma, n = 8). We compared our high resolution anatomical map to a functional spatial distribution map of TC inputs onto the L5 pyramidal cells in barrel cortex from our previous study using the lower resolution channelrhodopsin-2-assisted circuit mapping approach ('sCRACM') (Petreanu et al., 2009) (Figure 8D-F). The two methods reveal a similar concentration of TC input on the basal dendrites and proximal apical dendrites. However, sCRACM shows that the TC input strength on the apical dendrites in L4 and L3 is stronger than would be predicted from the anatomy. This difference suggests that synapse strength and/or electrotonic properties of dendrites are additional contributors to functional TC synaptic strength in L5 pyramids.

We next evaluated whether there was preferential targeting of certain dendritic branches by TC input. Such clustering of input on specific dendritic branches has important implications for integration of synaptic input and synaptic plasticity (Wei, 2001; Ariav et

al., 2003; Polsky et al., 2004; Losonczy and Magee, 2006), but has been difficult to evaluate because of a lack of available techniques. At a coarse level it was noticeable that L5 cells do not receive TC input uniformly, with some branches receiving denser input than others (see Figure 4). To quantify the uniformity of TC input we measured the number of TC synapses as a function of dendritic length and plotted these two parameters. If all branches have an equal probability of receiving TC input then there will be a very close relationship between dendritic branch length and TC synapse number. If not then 'TC preferring' or 'non-preferring' branches (e.g. blue vs. red branches in Figure 9A) will generate scatter away from the line of unity in a branch length vs. TC synapse number plot. To quantify this, we plotted the number of synapses vs. branch length from the eight reconstructed neurons (Figure 9B, closed circles) and overlaid that with the confidence interval determined from a simulated random distribution (Figure 9B, dotted lines, 95% confidence level). This analysis shows that the experimental data set contains branches with an excess of TC synapses (red symbols) and a fraction with a lack of TC synapses (blue symbols) compared to the random distribution. We also evaluated preferential dendrite targeting by the TC input by plotting the normalized histogram for TC synapse density for individual dendritic branches, comparing the simulated data set for randomly distributed synapses with the experimental data set. This analysis showed that compared with the randomly distributed synapses, the experimental data exhibited a larger fraction of branches at the extremes of the distribution, i.e. that have no TC synapses or a high TC synapse density (Figure 9C). These findings therefore indicate that TC afferents do not have equal preference for all dendritic branches but, instead, are biased toward a subset of preferred dendritic branches on L5 pyramidal neurons.

We further examined the spatial distribution of TC synapses, focusing on the distribution within individual dendritic branches. We found that at this level TC synapses are not regularly distributed, but show clustering. The mean distance between neighboring TC synapses across all dendritic branches was 5.6 ± 7.8 μm, compared with a mean distance of 14.24 ± 2.83 μm for regularly distributed synapses. The distribution of TC synapses within dendritic branches is different from a simulated random distribution (Figure 9D; cumulative probability distribution of nearest neighbor distances significantly different, p

= 2.5 x 10^{-77}, KS statistic = 0.20)**.** TC inputs from VPm onto L5 pyramidal cells occur primarily within L4, L5B and L6 (Bernardo and Woolsey, 1987; Bureau et al., 2006; Oberlaender et al., 2011b) and Figure 4. Therefore, it is possible that the clustering of TC synapses may be a layer-specific phenomenon rather than being specific to TC input. To test this, we compared the KS statistics for difference between clustering of TC and simulated random synapses on dendrites in L5 only with that for dendrites for the entire dendritic tree. If clustering was indeed a result of laminar organization of TC synapses, then the KS statistic would be significantly lower in value for TC input on L5 only compared with all dendrites. However, no difference was observed for the 8 neurons (data not shown), excluding such a laminar-specific effect as an explanation for the observed clustering of TC synapses.

There is evidence for cross-talk between synapses within ~5 μm on the same dendrite that enhances the expression of synaptic plasticity (Harvey et al., 2008; Takahashi et al., 2012), and which would be particularly significant for synaptic inputs of the same type. We therefore calculated the number of TC synapses that have one or more neighboring TC synapse(s) on the same dendrite within 5 μm. We found that 67.9 ± 5.4% of TC synapses have at least one neighboring TC synapse within 5 μm, whereas a value of 51.2 ± 5.5% would be predicted for a random distribution. Thus, the clustering of TC input is predicted to enhance synaptic plasticity between TC synapses onto the same dendritic branches in L5 pyramidal neurons. To further quantify the spatial clustering, we compared the clustering coefficient of TC synapses for the experimental data set and the random distribution across a range of distances (see Material and Methods). This analysis shows that there is a consistently greater degree of clustering for the experimental data set at distances of 5 – 15 μm (Figure 9E). Therefore, these analyses demonstrate significant clustering of TC synapses within dendritic branches, in addition to preferential targeting of certain branches by the TC input.

Discussion

Here we characterize the use of AT for the high-resolution analysis of the distribution of synaptic input from an identified presynaptic source onto an identified population of

postsynaptic neurons. We define the accuracy of synapse detection by AT using correlative EM imaging of the same serial sections. We go on to quantify for the first time the subcellular distribution of TC inputs onto the dendritic tree of L5 pyramidal neurons in mouse barrel cortex using LSAT. From this analysis we find that TC input targets specific dendritic branches and further that within branches it is clustered in a manner predicted to enhance plasticity and integration of TC input to L5.

Accuracy of Synapse Detection by AT

Previous work using AT (Micheva and Smith, 2007; Micheva et al., 2010) did not quantify the accuracy or the reliability of the technique for synapse detection. A recent theoretical study (Mishchenko, 2010) suggested that an accuracy of up to 80% can be achieved using AT alone when using staining of two synaptic markers (pre- and postsynaptic) in addition to labeling of pre- and postsynaptic neurons. To address this issue experimentally, we compared for the first time the accuracy of synapse detection using AT with EM synapse detection on the same serial sections. We show, under our light imaging conditions of 200 nm isotropic resolution with three color labeling of synapses, that we achieve ~78% accuracy of synapse assignment of TC inputs to L5 pyramidal cell dendrites. When we compared this value to the predicted accuracy of TC synapse detection using simulations, we found it to be slightly better than the predicted 70% accuracy. This difference is likely due to differences in the detection criteria used in the simulations. In the simulations we employed a simple and model-independent detection method for synapses, whereas in the experimental data synapses are detected as contacts between axons and spines, and this additional criterion has previously been shown to decrease the false-positive detection of synapses by optical image-based methods (Mishchenko et al., 2010; da Costa and Martin, 2011). Also the relatively large size of TC synapses likely further increases the accuracy of their detection (da Costa and Martin, 2011). Finally, in contrast with the assumptions in the simulation, we also observed a consistent gradient of synaptophysin staining towards the postsynaptic spine (Figure 10), reflecting an increasing concentration of synaptic vesicles with increasing proximity to the active zone. This feature provides an additional improvement in the accuracy of TC synapse detection.

We used synaptophysin immunostaining as our synaptic marker for synapse detection in AT, which labels all presynaptic vesicles and is one of the most abundant synaptic vesicle-specific proteins (Takamori et al., 2006; Micheva et al., 2010) (Figure 6). An alternative would have been to use labeling by a synapse associated protein (e.g. PSD-95, piccolo, bassoon) because this may increase the accuracy of synapse detection by directly labeling the pre- or postsynaptic membrane. However, immunostaining against synapse-specific proteins such as PSD-95, piccolo and bassoon has been shown to produce a relatively high false negative rate (Micheva et al., 2010). Furthermore, in our hands we were unable to quantify the accuracy and reliability of PSD-95 immunostaining with correlative EM because the antibodies were unsuitable.

In addition to quantifying false positive rates for AT, we also provide an estimate of the false negative rate for synapse detection by AT. The use of correlative EM imaging on the same sections in which light imaging was performed, allowed us to determine the number of TC synapses that we didn't detect by AT alone. These were characterized by the presence of a tdTomato- and synaptophysin-positive presynaptic terminal (detected by light imaging) and a PSD detected in EM, but for which no postsynaptic spine GFP signal was detected by light imaging. This evaluation provided an estimate of ~15% false negative rate. However, this is likely to be an overestimation because AT uses 3D data from serial sections and a considerable proportion of synapses are detected in at 2 adjacent sections when using AT (e.g. Figure 3). Yet, in the analysis of false negatives only one section was used because of the difficulty in reconstructing the EM images in 3D due to the relative poor membrane preservation produced using the correlative EM preparation protocol. Thus it is likely that the false negative rate for TC synapse detection is lower than 15%. Furthermore, it is likely that the undetected (false negative) synapses are small in size and therefore likely to be functionally less relevant (Matsuzaki et al., 2001) (Murthy et al., 2001).

Thus, we find that AT can detect TC synapses with 79% accuracy and more than 85% reliability under our three-color imaging conditions. Compared to the previously reported

best accuracy of 30-50% using traditional light microscopy (Sorra and Harris, 1993; da Costa and Martin, 2011), AT represents a significant improvement in synapse detection.

The Utility of LSAT for Large-Scale Mapping of Defined Synaptic Input

The quantification of the accuracy and reliability of TC synapse detection allowed us to map TC synapses in a large volume of reconstructed mouse barrel cortex (1.00 x 0.83 x 0.21 mm^3). The imaging of such a large volume at such resolution presented a number of technical challenges. For example, this approach necessitated the design of a custom imaging system with customized autofocus to enable reliable imaging of the 85,920 individual sections in three colors over a period of 877.5 hrs of continuous imaging. In addition, custom software was required for the appropriate alignment of the images. One of the major challenges not fully resolved is the full segmentation of images to enable automated tracing of individual neurons and the automated detection of synapses. The tracing of neurons and detection and assignment of TC synapses were performed manually. More work on these aspects of image analysis will be required to facilitate a higher throughput workflow for future projects.

Functional Consequences of TC Synapse Clustering on L5 Pyramidal Neurons

Specialized spatial domains on dendrites preferentially targeted by certain inputs have been hypothesized to act as separately functional units for integration and plasticity (Poirazi et al., 2003a; 2003b), providing a first layer of internal computation for neurons (Schiller et al., 2000). The consequence of such dendritic branch-level processing is that individual branches can coordinate its inputs using plasticity and can act as a computational unit representing different input features in the network (Hausser and Mel, 2003; Losonczy et al., 2008). Although there is some experimental support for this concept, it is not known whether synapses from the same presynaptic input can participate in local dendritic interactions to produce functional dendritic computational units representing related information from the same presynaptic source. We show that TC inputs have appropriate anatomical features to participate in such local functionally-related dendritic integration and plasticity. We show that TC inputs, carrying topographically organized sensory input preferentially target certain L5 dendrites and

cluster in a manner likely to increase their functional integration and promote plasticity. Such input-specific clustering is predicted to be a powerful mechanism for circuit development promoting topographical organization of ascending sensory input.

During development, such functionally clustered units of TC input would likely be promoted by synaptic plasticity in response to experience. TC axons during development are constantly edited at a high rate (35 μm/hr) (Portera-Cailliau et al., 2005) to revise their synaptic connectivity in a mechanism depending on activity. Long-term synaptic plasticity during early postnatal development has been proposed as a functional maturation cue at TC synapses (Isaac et al., 1997; Kidd and Isaac, 1999; Feldman et al., 1999; Cline, 2001). Induction of long-term potentiation (LTP) at one synapse enhances the likelihood of LTP induction at neighboring synapses within ~5 μm (Govindarajan et al., 2006; Harvey and Svoboda, 2007; De Roo et al., 2008). This process provides a candidate mechanism to promote the clustering we observe for TC synapses in which there is ~35% increase in the incidence of TC synapses located within 5 μm of each other (compared to a random distribution; see Figure 9D). Further support for this idea comes from findings showing that synapses are spatially organized on a fine scale to promote synchronized activity, development of which relies on NMDA receptor activation (Kleindienst et al., 2011; Makino and Malinow, 2011). In addition, a recent in vivo study shows that dendritic spines newly generated during learning form clusters (Fu et al., 2012).

In addition to the local clustering, we also find that TC inputs onto L5 pyramidal neurons primarily target basal dendrites proximal to the soma (Figure 8). Together with spatial clustering, this proximal dendritic targeting likely further promotes a reliable input-output function for the TC projection, making the TC input strong and driving in L5. We found that TC inputs also target proximal apical dendrites of L5 pyramidal neurons within L4. Although these synapses are present at a somewhat more distant location from the soma than the basal dendritic targeting inputs, the large diameter proximal apical dendrites exhibit reduced attenuation of these synaptic responses making this part if the TC input also relatively strong. This is borne out by functional mapping data using sCRACM

(Petreanu et al., 2009), which shows a larger functional TC response mediated by the TC input to L5 pyramidal cells onto the proximal apical dendrites in L4 than expected by the anatomic distribution we observe.

Conclusion

In summary, we describe the use of AT for large scale reconstruction of defined synaptic input onto defined postsynaptic neurons. We quantify the accuracy and reliability of synapse detection by AT for the first time and then show that it is suitable for the high resolution mapping the TC input onto L5 pyramidal neurons in a large volume of reconstructed barrel cortex. We find that TC synapses preferentially target certain dendrites and that TC synapses cluster in a manner predicted to enhance dendritic integration and plasticity. We anticipate that LSAT will be a highly useful tool for the quantitative mapping of connectivity in the brain, a key activity necessary for understanding information processing.

Material and Methods

Animals:

All experimental protocols were conducted according to the United States National Institutes of Health guidelines for animal research and were approved by the Institutional Animal Care and Use Committee at the Janelia Farm Research Campus.

Population-specific fluorescent labeling of neurons:

For the LSAT experiment we used Thy1-YFP (type H) transgenic mice, in which L5 pyramidal neurons are sparsely labeled with YFP (Feng et al., 2000). In these mice (approx. 5-6 months of age) we labeled TC synapses in the barrel field of somatosensory cortex by transducing VPm neurons with tdTomato. This was achieved by stereotaxic injection of adeno-associated virus (AAV; serotype II) expressing tdTomato under the CAG promoter and allowing ~4 weeks to achieve high levels of expression, as previously published (Petreanu et al., 2009; Hooks et al., 2011). In brief, after making a small incision in the scalp, we injected virus into the VPm of the thalamus through the thinned skull (1.45 mm posterior, 1.6 mm lateral to the Bregma and 3.1 mm deep from the pial surface). About 100 nl of viral suspension was injected through a pulled glass micropipette (Drummond, Broomall, PA). Successful targeting of VPm was confirmed by the distribution pattern of axons in S1 under low power fluorescence imaging (MVX10, Olympus, Tokyo, Japan). As previously described, neurons in L4 and L5B are the main recipients of ascending input from VPm (Bureau et al., 2006; Petreanu et al., 2009).

For the correlative light and EM study we labeled L4 neurons with GFP and TC axons and terminals using AAV-tdTomato injected in VPm. To achieve L4 specific gene delivery, we stereotaxically injected an AAV virus encoding FLEX-reversed GFP (Schnutgen et al., 2003; Atasoy et al., 2008) into L4 of a six3-CRE transgenic mouse line that expresses CRE in L4, but not other neocortical layers (Liao and Xu, 2008).

Tissue preparation, resin embedding and ultra-thin sectioning:

For correlative AT and EM, ultrathin sections had to be prepared without significant loss of fluorescence or immunoreactivity, yet with sufficient structural preservation to identify synapses unambiguously. We found the following fixation and embedding protocol meets our requirements. 20 - 30 days after the stereotaxic virus injection, animals were transcardially perfused with ~200 ml of fixative (4% paraformaldehyde, 0.2% glutaraldehyde in 0.1 M sodium cacodylate buffer). Brains were further incubated in fixative for ~2 hours before being extracted (Knott et al., 2009). Brains were then rinsed overnight with sodium cacodylate buffer and 300 μm thick sections prepared by vibratome (Leica, VT1200). The sections were then washed in cacodylate buffer and post-fixed with 0.001% osmium for 1 hour at 4^{o}C for correlative EM. Samples were then dehydrated by serial incubation in 30, 50, 70 and 95% ethanol and then embedded in LR White using a chemical accelerator (Electron Microscopy Sciences, Hartfield, PA) in a low temperature embedding system at -20^{o}C (Leica AFS2, Leica, Buffalo Grove, IL). Embedded tissues were sectioned at 60 nm thicknesses with an ultramicrotome (Leica, Buffalo Grove, IL) and collected on pioloform-coated Ø25-mm coverslips (Electron Microscopy Sciences, Hartfield, PA), so that the sections could be readily separated from the coverslip for subsequent EM. Sections mounted on pioloform film were separated from the coverslip with diluted hydrofluoric acid aqueous solution, transferred to an EM grid and stained with 7.5% uranyl acetate and Sato's lead solution (Sato, 1968).

For LSAT imaging, brains were fixed by transcardial perfusion with 4% paraformaldehyde, sectioned at 300 μm, embedded in LR white and 200 nm serial sections prepared and mounted on gelatin-coated 25 x 75 mm rectangular coverslips (Electron Microscopy Sciences, Hartfield, PA) using a Histo Jumbo knife (Diatome, Switzerland).

Immunohistochemistry:

Ultrathin sections were immunostained the following antibodies: synaptophysin (1:200, Synaptic System, Göttingen, Germany), DsRed (1:500, Clontech, Mountain View, CA), GFP (1:2000, abCam, Cambridge, MA) and visualized using fluorescence-tagged

secondary antibodies. Immunohistochemistry was conducted essentially as previously described (Micheva and Smith, 2007).

Light and electron microscopy:

For light microscopy we used a Zeiss Observer microscope (Zeiss, Göttingen, Germany) modified with a custom-built autofocus system. This system measures the distance between the objective and the sample surface with an auxiliary IR beam, and the sample is positioned within 100 nm of a preset target distance. Sample variation can cause changes in the distance measurement, so the edge sharpness within each image is analyzed after each acquisition. If poor edge sharpness is detected, the system acquires a z-stack of 15 images, and analyzes these images to determine the position of best focus for the current sample area; this newly determined target distance is used to reimage the field. A 100x, 1.45 N.A., oil-immersion objective was used for all the AT imaging. For LSAT, we collected 20 x 12 images in three colors, overlapping one another by 10%, from each of 1074 sections.

For EM, samples were imaged using automated EM acquisition software, Leginon (Suloway et al., 2005) in a Tecnai Spirit transmission electron microscope (FEI, Netherland) at 48,000x or 29,000x magnification. Images were registered at a coarse level using translational and rotational transformation in *TrakEM2* (Cardona et al., 2010) and were then aligned further using custom software. For image alignment between light microscope and EM images, images were manually aligned using mitochondria as common feature points in TrakEM2.

For LSAT, light microscopic images were aligned using custom software. The custom alignment software assigns one affine transform to each image in the stack using the following sequence of operations. All images are histogram equalized to enhance image contrast. A set of image-to-image correspondence point pairs is found for each pair of images that overlap in the same or adjacent z-plane. These points are found by maximizing normalized cross correlation in the overlap region using fast Fourier transforms (FFTs) and then refining the matching using a deformable mesh of triangles.

The triangle centroids become the correspondence points. Next the set of all image-to-image correspondence point pairs is scanned for mutual connectivity, which determines the set of transforms $\{T_i\}$ sought. A large system of linear equations is constructed to express that the correspondence point pairs should map to the same place in the common global space. After solving the system using conventional matrix methods, the residual correspondence point displacements can be used to express a fit accuracy. In the current case, the R.M.S. error for the whole stack is roughly 8 pixels. The shape of the error distribution is approximately Poisson, and much of the high error tail comes from very sparse regions at the periphery of the stack where matching is poorer but precisely because content is lacking. The interesting and denser regions are sufficiently well aligned for manual tracing.

Dendrite Segmentation and TC Synapse Assignment:

The dendritic structure of 8 neurons was manually traced using *Knossos* (Helmstaedter et al., 2011). Putative synapses were assigned on AT images based on the profiles of fluorescence. TC axons were recognized based on overlapping fluorescence of tdTomato and synaptophysin immunolabeling, and postsynaptic partners of cortical neurons were identified by the YFP signal. Therefore, putative TC synapses in AT were assumed to have three-color channels overlapping. In addition, we used the gradient of synaptophysin signal towards the synapse as a further confirmation of the direction of synapses (Figure 10). The asymmetrical accumulation of synaptic vesicles near the active zone produces a gradient of blue synaptophysin immunostaining intensity that increases towards the active zone. In addition, because of the increasing abundance of synaptic vesicles, there is a concomitant reduction in the amount of the presynaptic cytosolic fluorescent label (tdTomato) with increasing proximity to the active zone. These effects combine to produce a gradient of increasing synaptophysin staining (blue) and decreasing cytosolic fluorescent protein (red) with increasing proximity to the synaptic contact enhancing TC synapse detection reliability. TC synapses were assumed to be axo-spinous (Benshalom and White, 1986; White and Rock, 1980; da Costa and Martin, 2011), therefore, thalamic afferents on large green structures, which are likely a part of dendritic shafts or soma, were not assigned as putative TC synapses. The accuracy of TC synapses

assignment was evaluated with the correlative EM images. Since synaptic vesicles in presynaptic terminals were not visible under our correlative EM conditions, synapses in these EM images were defined by the presence of an electron dense postsynaptic density (PSD) structure. To avoid experimenter-based bias, the accuracy of synapses assignment both in light and electron microscopy was assessed independently by two scientists. Dendritic morphology was quantified using 3-dimensional Sholl analysis. The number of intersections between dendrites and concentric spheres centered at the center of the cell body were counted at various diameters using custom software.

Simulations:

For the simulations of accuracy of detection using AT (shown in Figure 7) we used the serial electron microscopy data from (Mishchenko et al., 2010). In this data, all synapses, presynaptic boutons, and postsynaptic spines were marked automatically and then verified manually as described in (Mishchenko, 2010). Only synapses between well defined spine heads and axonal boutons were kept for the calculation. These constituted a dataset of 250 well defined synaptic contacts. For each individual presynaptic bouton we constructed in *Matlab* (Mathwork, MA) the distance transform for the associated spine head using the fast anisotropic 3D distance transform Matlab function *bwdistsc* (Mishchenko, "3D Euclidean Distance Transform for Variable Data Aspect Ratio", Matlab Central Website, 2007). Thus, the distance transform assigned the distance from each presynaptic bouton to each reference spine head. Using these constructed distance transforms we calculated, for each synaptic contact and each light imaging resolution, the total number of different vesicle clouds located within that resolution limit away from associated spine head. If the number of such "proximal" vesicle clouds is greater than one, that creates a possibility for incorrectly assigning a presynaptic axon to a postsynaptic spine thus falsely identifying a synaptic contact. A vesicle cloud was assumed to contribute to the fraction of false-positives at resolution *d* if any of its vesicles were found to be within the distance *d* away from a spine head. Using this analysis, we calculated the probability of false-positive associations for each synapse at different light imaging resolutions. Specifically, if the total number of axonal boutons proximal to a reference spine at distance *d* was *n_spn(d)*, then the probability of false-positive

associations in these settings was calculated using the following formula: *p_spn(d)=(n_spn(d)-1)/n_spn(d)*. The overall false-positive error rate was then evaluated as the average over all the individual synapses.

To test whether the observed distribution pattern of detected TC synapses can be generated by chance alone (data shown in Figure 9), we built model neurons that have a random distribution of synapses on the same segmented dendritic structure of the eight reconstructed L5 pyramidal neurons. For each of the eight neurons, we did this by randomly distributing the same number of TC synapses as measured experimentally on a line the length of the total dendritic path length. The line was then broken up and reconstructed back into the real dendritic structure of the neuron to produce a random distribution of TC synapses. For each neuron this simulation was performed 1000 times.

Clustering coefficient:

Clustering coefficient was calculated using graph theory (Bullmore and Sporns, 2009), defining two synapses as 'connected', when they are within the defined neighboring distance. The clustering coefficient of each synapse (Figure 9E) was then calculated as the ratio of the number of connected pairs between neighboring synapses to that of all possible edges within the neighborhood. Clustering coefficients were averaged across all eight reconstructed neurons and compared to those calculated for simulated neurons with random synapse distributions. The difference in clustering coefficient between experimental and simulated data sets was compared using a KS test.

Statistics:

To compare the experimentally observed TC synapse distribution to the random distributions, a non-parametric distribution-free Kolmogorov-Smirnov test (KS test) was used. Paired t-tests were used to examine whether the TC synapse distribution pattern is significantly altered by restricting the analysis on the branches within L5. $p<0.05$ was considered as statistically significant. All errors shown in the text as well as in the figures are standard deviation.

Conflict of interest statement

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Figure Legends

Figure 1. Labeling and Imaging Thalamocortical Synapses on L4 or L5 Barrel Cortex Neurons using Array Tomography and Electron Microscopy

(A1) Low power fluorescence photomicrograph of tdTomato-expressing TC axons and terminals in a fixed section of barrel cortex prepared from a 5-6 month old mouse in which AAV-tdTomato was stereotaxically injected in VPm 20-30 days earlier (inset left shows schematic of VPm injection). **(A2)** Low power fluorescence photomicrograph of GFP fluorescence in neurons in L4 barrel cortex in a fixed section from adult six3-CRE mouse in which AAV-FLEX-revGFP virus was injected into L4, 20-30 days earlier. **(A3)** Low power fluorescence photomicrograph of GFP fluorescence in L5 pyramidal neurons in fixed section from a 5-6 month old Thy1-YFP_h mouse. **(B)** Schematic of LR white embedding and ultrathin serial sectioning procedure. **(C)** Schematic of immunostaining and light imaging of serial sections. **(D1)** Schematic of light and electron microscopic imaging on the same sections to assess accuracy of synapse detection and annotation with array tomography (performed on VPm-L4 only). **(D2)** Reconstructed L5 pyramidal neuron and annotated TC synapses from large scale array tomography experiment (VPm-L5 only).

Figure 2. Large Scale Array Tomography Images from Barrel Cortex of Thy1-YFP_h Mouse.

(A) Maximum intensity projected YFP image from the aligned image stack obtained from 1074 serial sections. **(B)** Maximum intensity projection of 300 serial sections (=60 μm thick) from the box in A showing YFP-expressing L5 pyramidal neurons. **(C)** Orthogonal y-z view of (B). **(D)** Close up of box in (B), 150 serial sections (=30 μm thick) showing VPm axons/terminals in red, YFP-expressing L5 dendrites in green and synaptophysin staining in blue. **(E)** The tracings of the eight reconstructed L5 neurons superimposed on the max projection YFP image. Scale bars: 100 μm (A), 50 μm (B), 20 μm (D), 200 μm (E).

Figure 3. TC Synapse Detection by AT.

High power view of a section of dendrite of an YFP-expressing L5 pyramidal neuron with four example thalamo-recipient spines indicated (left panel; 3-D volume rendering of 13 serial sections = 2.6 μm thick). Right panels: images from three serial sections showing spine (YFP signal, green), TC terminal (tdTomato, red) and synaptophysin staining (blue) for each of the four indicated example TC synaptic contacts. Yellow arrows indicate locations of identified synaptic contacts (note that for synapses 2 and 4, the synaptic contact is detected in two of the images).

Figure 4. Tracings of the Eight Reconstructed L5 Pyramidal Neurons.
(A) Enlarged image of the reconstruction of neuron 1 including positions of all TC synapses (red stars; scale bar = 100 μm). **(B)** Each dendritic branch is color-coded for TC synapse density (scale bar = 200 μm).

Figure 5. Preparation Conditions used for Correlative Electron Microscopy Preserve Antigenicity While Allowing Reliable EM Detection of Synapses
(A-C) Comparison of number of synaptophysin-positive punctae using different fixation protocols. Representative light microscopic images of synaptophysin immunostaining in sections from brains fixed using the LSAT protocol (A) and the correlative EM protocol (B). Quantification of synaptophysin-positive punctae in the two conditions (C). **(D-F)** Comparison of the number of PSDs detected in EM images under the two fixation conditions. Representative electron micrographs from sections from brains fixed using a traditional EM fixation protocol (D) and the correlative EM fixation protocol (E). Red arrows show identified synapses. Quantifications of number of synapses detected PSD under traditional EM conditions and from four independently prepared samples using the correlative EM conditions (F).

Figure 6. Combined Electron and Light Microscopy to Determine the Accuracy of Synapse Detection by Array Tomography.
(A-C) Transmission EM (A) and light microscopy (B) images from the same section of tissue, and the two images superimposed (C). Yellow contours in all three images show mitochondria that are used as fiducial markers for aligning images. Arrowheads indicate

predicted TC synapses based on the light microscopic image, colors of arrowheads show true-false evaluation based on the EM image (as depicted in D). (C1-C5) Close up images of TC synapses predicted by light microscopy. **(D)** Schematics of synapse assignments based on combined EM and light microscopy. **(E)** Quantitation of different synapse assignments based on combined EM and light microscopy (evaluation of 322 putative TC synapses).

Figure 7. Simulation of Accuracy of Synapse Detection by AT.

(A) Simulated AT images from a previously segmented EM image data set (from (Mishchenko et al., 2010)). (A1) an example EM image; (A2) same image segmented into pre- and postsynaptic structures; (A3) same segmented image with 90% of structures removed to mimic the sparseness of fluorescent labeling of the AT images; (A4) same segmented image blurred to produce the same resolution as in the light microscopy in AT. Red indicates presynaptic structures, green postsynaptic, blue synaptophysin and red arrow indicates a synapse. Scale bar = 1 μm **(B)** Close-up of a simulated synapse showing postsynaptic structure (green) and simulated synaptophysin staining (blue). Note that the predicted synaptophysin staining exhibits an increasing intensity gradient towards the synaptic contact as found experimentally (Figure 10; Scale bar = 0.5 μm). **(C)** False positive rate in simulations of AT using different light imaging isotropic spatial resolutions. Black dashed line indicates resolution of the light imaging in the current study. Blue dotted line is an extrapolation of simulated data to infinite resolution.

Figure 8. LSAT shows that TC Synapses are Concentrated on Proximal Basal Dendrites of L5 Pyramidal Neurons.

(A) Three dimensional Scholl analysis of dendritic structure of reconstructed L5 pyramidal neurons, and **(B)** Number of TC synapses as a function of path distance from center of soma. **(C)** Average density of TC synapses from all annotated neurons (aligned relative to cell bodies; false color scale) represented on top of all dendrites from all the traced neurons (white lines, superimposed; scale bar 100 μm). (**D, E**) Spatial distribution of the functional TC input mapped using sCRACM (Petreanu et al., 2009) (D, false color

scale; reduced to 80% of size to compensate for the tissue shrinkage during LSAT processing) and distribution of TC synapses detected by LSAT (E, average from all annotated neurons aligned relative to pia; scale bar 100 μm). **(F)** Overlay of TC laminar distribution measured by sCRACM and LSAT.

Figure 9. Clustering of TC Input on Dendrites of L5 Pyramidal Neurons

(A) Example of dendritic branches of the same L5 pyramidal cell (from dotted box in Figure 4B) showing different densities of TC synapses. **(B)** The relationships between length of dendritic branch and number of TC synapses for one of the reconstructed L5 neurons. Solid line is the line of unity; dashed lines denote 95% confidence intervals from simulated randomly distributed synapses. Outliers are color-coded in red (more TC synapses than expected compared to random) or in blue (fewer than expected compared to random). **(C)** Histogram of TC synapse density for all dendritic branches from all reconstructed neurons. Insets: expansions of peak and tail of the distribution as indicated by the dashed boxes. **(D)** Averaged cumulative distributions for nearest neighbor distances of TC synapses, for experimental data (red, eight reconstructed neurons) and for simulations of 8000 model neurons with randomly distributed synapses (black) **(E)** The mean clustering coefficient for TC synapses at various spatial thresholds from all eight reconstructed neurons (red line) and for simulated randomly distributed synapses (black line). Bars (right y-axis) show statistical results using a KS test to determine the fraction of simulations there are significantly different from the experimental data set, which shows that >80 % of the simulations with random synapses have significantly different clustering from the experimental data set across the spatial threshold range.

Figure 10. Distribution of GFP, tdTomato and Synaptophysin Staining at TC Synapses.

(A) Three-color image of an example TC synapse (green is postsynaptic GFP, red is presyanptic tdTomato, blue is presynaptic synaptophysin stain). **(B)** Pixel intensity map for each color channel for image in A (left) and plot of pixel intensity averaged across all pixels vertically (right) around a TC synapse. **(C)** Pixel intensity map for each color

channel averaged for 30 TC synapses. Note that the synaptophysin signal is skewed towards the synapse but the tdTomato signal is not. Images shown are ~1.1 x 0.4 μm^2.

Supplementary Material:

Supplementary movie. Three-dimensional volume rendering of LSAT from Figure 2B.