Fluorescence-based quantitative synapse analysis for cell-type specific connectomics

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Title: Fluorescence-based quantitative synapse analysis for cell-type specific connectomics

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DAK was responsible for all aspects of the in vivo construct expression and quantitative synaptic analysis and writing of the manuscript. EP contributed to synaptic quantitation and input analysis. CAT was responsible for design and generation of FAP-post constructs. JL performed in vivo imaging. DSA contributed to the cloning of Cre-dependent AAV vectors. MPB was responsible for design and creation of the postsynaptic targeting constructs and contributed to fluorescence image analysis, and manuscript preparation. ALB was responsible for construct and experimental design, data acquisition and analysis, and writing of the manuscript.

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Abstract

Anatomical methods for determining cell-type specific connectivity are essential to inspire and constrain our understanding of neural circuit function. We developed genetically-encoded reagents for fluorescence-synapse labeling and connectivity analysis in brain tissue, using a fluorogen-activating protein (FAP)- or YFP-coupled, postsynaptically-localized neuroligin-1 targeting sequence (FAP/YFPpost). FAPpost expression did not alter mEPSC or mIPSC properties. Sparse AAV-mediated expression of FAP/YFPpost with the cell-filling, red fluorophore dTomato (dTom) enabled high-throughput, compartment-specific detection of putative synapses across diverse neuron types in mouse somatosensory cortex. We took advantage of the bright, far-red emission of FAPpost puncta for multichannel fluorescence alignment of dendrites, FAPpost puncta, and presynaptic neurites in transgenic mice with saturated labeling of parvalbumin (PV), somatostatin (SST) or vasoactive intestinal peptide (VIP)-expressing neurons using Cre-reporter driven expression of YFP. Subtype-specific inhibitory connectivity onto L2 neocortical pyramidal (Pyr) neurons was assessed using automated puncta detection and neurite apposition. Quantitative and compartment-specific comparisons show that PV inputs are the predominant source of inhibition at both the soma and the dendrites and were particularly concentrated at the primary apical dendrite. SST inputs were interleaved with PV inputs at all secondary- and higher-order dendritic branches. These fluorescence-based synapse labeling reagents can facilitate large-scale and cell-type specific quantitation of changes in synaptic connectivity across development, learning, and disease states.

Significance Statement

High-throughput quantitation of synapse number and distribution can reveal principles of circuit function and their adaptive or pathological alterations. Molecular genetic, fluorescence-based approaches targeted to discrete cell types can enable automated detection and quantification of input-specific synapses in complex brain tissues. In addition, these tools present a low barrier to use within...
the neuroscience community through volumetric confocal analysis of tissue specimens. Here we evaluate inhibitory synapse distribution across layer 2 (L2) pyramidal neurons using postsynaptic expression of a previously characterized, neuroligin-based construct. We find that inhibitory inputs from fluorescently-labeled parvalbumin and somatostatin neurons are intermingled across the proximal dendrites, and that inputs from vasoactive-intestinal peptide neurons are rare for L2 pyramidal neurons.
The organization, number, and input identity of synapses onto a cell are critical determinants of neuronal activity. Although electrophysiological analyses of synaptic properties have provided a rich framework to build and test hypotheses about neural computations during sensation and behavior, these analyses cannot reveal broader principles of synaptic distribution across the neuron. Since alterations to synaptic function in select circuits and cell types are associated with autism, intellectual disability, psychiatric, and neurologic disease (Bayes et al., 2011; Sudhof, 2017), quantitative metrics about synaptic location, size, and input specificity are likely to provide key insights into how neural circuits are related to disease pathology.

Electron-microscopy (EM) provides nanometer resolution for ultrastructural identification of synaptic contacts and has been employed for brain-area and cell-type quantitative analysis (Bock et al., 2011; Briggman et al., 2011; Chandrasekaran et al., 2015; Kim et al., 2014); however, EM is hampered by technical demands of sample preparation, imaging time, data storage, and labor-intensive analysis that make comparisons across multiple individuals or conditions difficult. Recent studies have attempted to use EM for quantitative analysis of synapse organization between defined pre- and postsynaptic partners, but these computationally-intensive approaches are difficult to adopt and scale for broad use (Glausier et al., 2017; Kornfeld et al., 2017; Kubota et al., 2015; Vishwanathan et al., 2017). Fluorescence-based microscopy methods are an attractive alternative to EM, because light-microscopy facilitates faster acquisition of larger tissue volumes and enables use of spectrally distinct, genetically encoded fluorophores for discrimination of molecularly diverse cells and synapse types.

There has been great interest in developing tools and methodologies for synapse labeling using molecular, genetic, or histochemical techniques for light microscopy, including GFP-tagging synaptic molecules, GFP reconstitution across synaptic partners (GRASP) and array tomography (Chen et al., 2012; Fortin et al., 2014; Gross et al., 2013; Kim et al., 2011; Kinoshita et al., 2019;
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Martell et al., 2016; Micheva and Smith, 2007; Villa et al., 2016b). Fluorescence-based, sparse labeling of post-synaptic neurons in intact brain tissue has been especially helpful in this regard, as it reduces the analysis bottleneck that arises from broadscale immunohistochemical labeling of synapses from neurons intermingled in the analysis volume. However, high-throughput/volumetric synaptic analysis for individual neurons has not yet become routine, possibly due to low signal-to-noise and synaptogenesis or abnormal synapse stabilization associated with overexpression of synaptic tags (El-Husseini et al., 2000; Kim et al., 2011; Martell et al., 2016; Tsetsenis et al., 2014).

We sought to develop molecular genetic tools for comprehensive fluorescence labeling of postsynaptic sites across an individual neuron, in a complex tissue environment. We employed fluorogen-activating protein (FAP), a modified antibody fragment that emits in the far red upon binding of a small molecule ligand, a derivative of malachite green (MG; Szent-Gyorgyi et al., 2013), targeted to postsynaptic sites using the well-validated post-synaptic tag derived from the transmembrane and cytoplasmic region of mouse neuroligin-1 (NL-1) (Druckmann et al., 2014; Kim et al., 2011; Kwon et al., 2018).

Sparse, virus-mediated coexpression of FAPpost with the cell-filling fluorophore dTomato (dTom) showed broad, punctate labeling across distinct pyramidal cell compartments. Aided by automated image analysis, we quantitatively evaluated the distribution of inhibitory synapses identified from analysis of more than 90,000 synaptic puncta in neocortical pyramidal (Pyr) neurons in superficial layers of somatosensory cortex. Using comprehensive fluorescence-labeling of cell-type specific neurites in parvalbumin (PV), somatostatin (SST), and vasoactive intestinal peptide (VIP) Cre-driver transgenic mice allowed us to align FAPpost puncta to quantify inhibitory inputs for L2 Pyr neurons. This quantitative analysis revealed that PV inputs dominated the soma and the synapse-dense 1° apical dendrite, and that PV inputs had a moderately higher density than SST inputs across all L2 dendrites. VIP neurons only sparsely innervated L2 Pyr neurons. These studies help...
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establish a framework for a high-throughput analysis of synapse organization in brain tissue during health and disease.

Materials and Methods

All experimental procedures were conducted in accordance with the NIH guidelines and were approved by the Institutional Animal Care and Use Committee at Carnegie Mellon University.

Construct Design

FAPpost Cloning

To make the plasmid for packaging into AAV, post-mGRASP from Addgene (#34912 - paaCAG-post-mGRASP-2A-dTom) was modified by annealing oligos and inserting into BamHI and XhoI digested backbone to introduce an AgeI site (PostBamXhoF 5’GATCC CTT ACCGGT ATC TTA C and PostBamXhoR 5’ TCGAG TAA GAT ACCGGT AAG G). PCR was used to produce the Igkappa leader sequence, cmyc epitope and dL5** FAP (Szent-Gyorgyi et al., 2008; Szent-Gyorgyi et al., 2013; Telmer et al., 2015) for introduction into the BamHI and AgeI of the modified backbone (BamKappaF 5’ TATATA GGATCC ggcttggggatatcca ccatgg and dL5AgeSfiR 5’ TATATA ACCGGT ACCTCC ggccagacgccgcggc GGAGAG). The BamHI/HindIII fragment was moved to create pENN.AAV.hSyn.kappa.myc.dL5.POSTsyn.T2A.dTom.WPRE.BGH (Addgene FAPpost plasmid ID 105981). AAV1 serotype was produced by Penn Vector Core.

Fl-YFPpost and fl-FAPpost Cloning

For Cre-inducible expression, the kappa.myc.dL5.POSTsyn.T2A.dTom region was PCR amplified with primers containing BsrG1 and KpnI restriction sites (partial KpnI digestion was required) and ligated into digested pAAV-FLEX (fl; generous gift from Oliver Schluter) to produce pAAV-FLEX-hSyn-kappa-myc-dL5-POSTsyn-T2A.dTom.WPRE-SV40. PCR amplification was used to generate the SYFP2 (YFP) (Kremers et al., 2006) coding fragment (iGEM BBa_K864100) that was then SfiI digested to replace the FAP in the pAAV-FLEX resulting in pAAV-FLEX-hSyn-
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kappa-myc-dL5-POSTsyn-T2A-dTom-WPRE-SV40 (Addgene fl-FAPpost plasmid ID 105982; Addgene fl-YFPpost plasmid ID 105983). Constructs were packaged into AAV1 and produced by Penn Vector Core.

Animals

Experiments were performed on wild-type and transgenic reporter male and female mice on a C57BL6J background (Table 1). Cre recombinase lines used included Emx1-IRES-Cre (Jackson Labs stock #005628, Pvalb-2A-Cre (Jackson Labs stock #008069; (Hippenmeyer et al., 2005)), SST-IRES-Cre (Jackson Labs stock #013044; (Taniguchi et al., 2011) and VIP-IRES-Cre (Jackson Labs stock #010908; (Taniguchi et al., 2011)). Homozygous Cre-expressing mice were mated with homozygous Ai3 mice (Jackson Labs Stock #007903) to create heterozygous transgenic mice with eYFP- (YFP)-labeled SST, PV, or VIP interneurons. Pyr cells from at least three mice from each line were used to characterize FAPpost expression patterns.

Virus injection surgery

FAPpost virus (0.4 μL) was stereotaxically injected into barrel cortex through a small craniotomy (bregma -0.9, lateral 3.00, depth 0.5 mm) in isoflurane-anaesthetized mice aged postnatal day (P12-17) using a Hamilton syringe (Hamilton; Reno, NV), Stoelting infusion pump 597 (Stoelting; Wood Dale, IL, Model #53210), and custom injection cannulas (Plastics One; 598 Phoenix, AZ). Mice were treated once with ketofen (5 mg/kg, Sigma-Aldrich; 599 St. Louis, MO), then allowed to recover in their home cage until weaning (P21), when they were moved to a new cage with their littermates.

Fixed tissue preparation and immunohistochemistry

Seven to 15 days following virus injection, animals were anesthetized with isoflurane and transcardially perfused at mid-day using 20 mL phosphate buffered saline (PBS; pH 7.4) followed by 20 mL 4% paraformaldehyde in PBS (PFA; pH 7.4). Brains were removed, and postfixed overnight.
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at 4°C in 4% PFA before transfer into 30% sucrose cryoprotectant. After osmotic equilibration brains were sectioned (50μm thick section) using a freezing-microtome.

Free-floating brain sections containing dTom-expressing cells were washed using PBS before 30 minute room temperature incubation with malachite green (MG) dye (300nM in PBS; (Pratt et al., 2017)). MG-dyed sections were then rinsed with PBS before mounting on glass microscope slides with Vectashield fluorescent mounting media (Vector Lab; Burlingame, CA). Before MG dye application, a subset of brain sections underwent immunofluorescence staining. Brain sections from PV-Cre, SST-Cre, or VIP-Cre x Ai3 for saturated cell-type specific YFP labeling underwent GFP immunofluorescence staining to enhance YFP signal. These sections were first blocked (10% NGS, 0.1% TritonX, 0.1M PBS), and incubated for 48 hour at 4°C with anti-chicken GFP primary antibody (1:2000 dilution in blocking solution; Abcam AB13970; Cambridge, MA). Sections were rinsed with PBS, then incubated with Alexa488 anti-chicken secondary antibody (1:500, in blocking solution; Invitrogen A-11039; Carlsbad, CA). In the same manner, a subset of PV-Cre x Ai3 brain sections underwent bassoon immunofluorescence staining to visualize presynaptic release sites. These sections were blocked (10% DS, 0.2% TritonX, 0.1M PBS), then incubated overnight at 4°C with mouse anti-bassoon primary antibody (1:1500 in blocking solution; Enzo Life Sciences Assay Design VAM-PS003). Slices were rinsed with 0.2%PBST then incubated with Alexa405 anti-mouse secondary antibody (1:500 dilution in blocking solution; Invitrogen A-31553).

Confocal Imaging

FAPpost expression in L2/3 (~200-300μm below the pial surface) of the S1 barrelfield (S1BF) was confirmed by the presence of layer 4 barrels. Pyr cells were identified using morphological criteria, including the presence of a thick apical dendrite oriented toward the pial surface, pyramidal-shaped cell body, laterally projecting basal dendrites, a descending axon identified by its narrow diameter, and the ubiquitous presence of dendritic spines, particularly on higher-order branches. Only isolated Pyr cells (typically at the edge of the viral transduction zone) that exhibited dendritic dTom
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as well as punctate FAP signal were selected for imaging and quantitation. FAPpost and dTom expression were not always positively correlated, an effect that was unexpected given the construct design. dTom-expressing neurons that did not exhibit membrane-localized FAP fluorescence, or showed diffuse and low-intensity signal were excluded from analysis. Analyzed cells showed no significant relationship between FAPpost puncta intensity and calculated puncta density along the dendrite. In almost all cases, selected cells included the entire soma in the image dataset. Because cortical dendrites are > 200μm long and could lie outside the imaged area, only a fraction of the dendritic arbor was collected and analyzed.

Sections were observed under a LSM 880 AxioObserver Microscope (Zeiss), using 63x oil-immersion objective lens (Plan-Apochromat, 1.40 Oil DIC M27) with the pinhole set at 1.0 Airy disk unit. Maximum image size was 1024 x1024 pixels. Zoom factor was set to 1, corresponding to a voxel dimension of 0.13μm x 0.13μm x 0.32μm in X, Y, and Z directions. Selected cell bodies were centered in the field of view (135μm x 135μm). Up to 100 images with a Z-interval of 0.32μm and 50% overlap between optical sections were acquired per stack. Fluorescence acquisition settings were as follows: Alexa405 (excitation λ405, emission λ452, detection λ406-498), Alexa488 (excitation λ488, emission λ504, detection λ490-517), YFP (excitation λ514, emission λ535, detection λ517-535), dTom (excitation λ561, emission λ579, detection λ561-597), MG/FAP (excitation λ633, emission λ668, detection λ641-695). Optimal laser intensities for each channel were set for each cell independently, and images were collected to avoid pixel saturation. Well-isolated cells of interest were centered in the image frame and the Z-stack dimensions were set manually by tracking dTom labeled dendrites. Z-stacks typically ranged from 30-40μm for a given neuron. For experiments assessing bassoon immunofluorescence alignment with YFP-expressing PV neurites and FAPpost puncta on soma of transduced cells, image size was 1912 x 1912 pixels with a zoom factor was set to 2, corresponding to a voxel dimension of 0.05μm x 0.05μm x 0.32μm in X, Y,
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10 and Z directions. The total Z-stack depth typically ranged between 10-15μm starting from the brain section surface, where bassoon antibody penetration was most complete.

233 Cranial window construction for \textit{in vivo} imaging

234 One week after virus injection, mice were isoflurane anesthetized and heads fixed using a custom-made nose clamp. Eyes were covered with ointment, hair was removed with Nair, and scalp was disinfected with povidone iodine. Scalp and periosteum was removed, and skull surface roughened by scraping with a slowly rotating dental drill. A thin layer of Krazyglue was applied to the skull before a custom-made head bracket was attached in the right hemisphere using Krazyglue and dental cement (Lang Dental, 1223PNK). The skull was carefully thinned around a 3mm diameter circle centered above the left hemisphere S1BF using a dental drill (Dentsply, 780044). After extensive thinning, the loose bone flap was detached using a microforcep. A glass window composed of 3mm diameter glass (Harvard Apparatus, 64-0720) attached to 5 mm diameter glass (Harvard Apparatus, 64-0700) was mounted above the exposed brain. The window was sealed with 3MTM Vetbond\textsuperscript{TM}. A chamber wall was built around the window with dental cement. Ketoprofen (3 mg/Kg) was given subcutaneously.

Two-photon imaging

237 Mice were anesthetized with 1.5 \% isoflurane and mounted under a Femtonics FEMTO2D microscope. Layer 1/2 dendrites expressing dTom and YFPpost were visualized under a 63x objective using 950 nm excitation (Spectra-Physics Mai Tai HP; Santa Clara, CA) with simultaneous detection of dTomato and YFPpost using red and green PMTs, respectively. Single-plane 60x60μm (1000x1000 pixel) linescan (15x averaging) images were acquired using MES software (Femtonics, v.5.2878). The raw intensity matrix for each channel was converted to a grayscale image in MATLAB (MathWorks, R2017a). Channels were overlaid and brightness/contrast adjusted using Photoshop 6.0 (Adobe).

Electrophysiology
FAPpost-injected mice were sacrificed at age P20-25 by brief isoflurane anesthesia and decapitation. Coronal slices (350μm thick) were prepared in regular ice-cold artificial cerebrospinal fluid (ACSF) composed of (in mM): 119 NaCl, 2.5 KCl, 1 NaH2PO4, 26.2 NaHCO3, 11 glucose, 1.3 MgSO4, and 2.5 CaCl2 equilibrated with 95%/5% O2/CO2. Slices recovered in the dark at room temperature for 60 minutes before transfer to an electrophysiology rig where they were perfused with ACSF containing 1μM tetrodotoxin (Tocris, UK) to silence spontaneous activity. The injection site was identified by dTom fluorescent cell bodies using an Olympus light microscope (BX51WI). Pyr-targeted recordings (4-5 animals per group) were done in the absence of MG dye, since we were interested in whether in vivo expression of the FAPpost construct would influence synaptic function and MG was never applied before tissue fixation for anatomical analysis. Borosilicate glass electrode resistance was 4-8 MΩ. Electrode internal solution was composed of (in mM): 130 cesium gluconate, 20 HEPES, 0.4 EGTA, 2.8 NaCl, 5 Tetraethylammonium chloride (TEA-Cl), 4 Mg-ATP and 0.3 Na-GTP, pH 7.25-7.30, 280-290 mOsm. Trace amounts of AlexaFluor488 were included in the internal solution to confirm that targeted cells had pyramidal-like morphologies.

Electrophysiological data were acquired using a Multiclamp 700B amplifier (Axon Instruments; Foster City, CA) and a National Instruments acquisition interface (National Instruments; Austin, TX). The data were filtered at 3 kHz, digitized at 10 kHz and collected by Igor Pro 6.0 (Wavemetrics; Lake Oswego, Oregon). After forming a GΩ seal, negative pressure was applied to the cell to enter whole-cell mode, and following 2-3 minutes acclimation time, miniature excitatory postsynaptic currents (mEPSCs) were collected at -70 mV holding potential for 5 minutes. Holding potential was slowly raised to 0 mV over an additional minute, and following 1 minute acclimation time, miniature inhibitory postsynaptic currents (mIPSCs) were then collected. Traces were analyzed using MiniAnalysis (Synaptosoft Inc., NJ), with a 7pA minimal amplitude cut-off. One hundred randomly selected events for each cell (Pyr dTom- and dTom+) were used to create cumulative probability histogram.
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Image analysis

Bassoon alignment

PV-Cre x Ai3 mouse brain tissue sections containing FAPpost transduced cells were stained for bassoon, a presynaptic marker of vesicle-release active zones that localizes to both excitatory and inhibitory synapses (Richter et al., 1999). Images of the four different fluorescence channels (bassoon, PV/YFP, dTom, FAPpost) were arranged side-by-side in series for all optical sections containing the target soma. Analysis was restricted to the surface of a tissue section (~5 um from top) where bassoon antibody penetration was complete. In deeper regions of the tissue section, bassoon immunofluorescence was low to undetectable, making colocalization assessments unreliable.

First, bassoon puncta adjacent to the surface of a target dTom expressing soma were identified by an experimenter. Bassoon puncta sometimes extended across multiple optical sections. Bassoon colocalization at PV/YFP neurites was assessed by direct overlap of signal from the two channels. FAPpost puncta at the soma surface were counted as being associated with bassoon when puncta were aligned with <0.25 μm distance. A minimum of fifteen-bassoon puncta were assessed for colocalization/alignment for each soma analyzed. We independently examined the rate of FAPpost alignment with bassoon (to identify putative false positives) and PV, as well as PV alignment with bassoon and FAPpost in the same manner.

To assess dendritic FAPpost alignment with synaptic immunofluorescence, spiny dendritic segments running parallel to imaging plane (≥10μm in length) were manually assessed for alignment between FAPpost and bassoon across one to five flattened confocal sections. Most FAPpost puncta exhibited overlap with bassoon immunofluorescence within this sub-volume. Some FAPpost puncta without an apparent bassoon partner could extend beyond the thin volume assessed. In this minority of cases, additional adjacent optical sections at these specific locations were examined for bassoon signal to determine whether these FAPpost puncta were actual false positives.
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Carl Zeiss image files were imported into Imaris version 8.4 equipped with the Filament Tracer plugin (Bitplane; Zurich, Switzerland). The dTom cell fill was used to create a 3D cell-surface rendering using a combination of surface and filament objects. FAPpost puncta were first reconstructed as 3D structures using “surface objects” (to outline puncta borders) created using an estimated 0.5μm diameter. Due to imaging limitations, only puncta larger than 3 voxels (~0.024μm³) were counted, potentially undercounting very small synapses below this detection threshold. Large puncta that potentially reflected smaller, adjacent synapses were separated into multiple objects using the “split touching objects” function with the same estimated 0.5μm diameter. Thus, large puncta were potentially separated into multiple smaller synapses, a process that could increase the absolute number of detected synapses. Indeed, it is unclear for larger synapses whether these should be counted as a single synapse with multiple active sites and post-synaptic specializations (Tang et al., 2016), or combined into one large synapse (such as the giant synapses observed at the Calyx of Held). Puncta were digitally associated with the plasma membrane if their edges lay within 0.5μm from the soma surface or <1μm for spiny dendritic regions. Puncta 0.5μm below cell surface were attributed as cytosolic fluorescence and not included for analysis. Puncta “objects” were then converted into puncta “spots” (with automatic intensity max spot detection thresholds and a 0.5μm estimated-diameter) using “surface object” centroids in Imaris.

Puncta quantification

Puncta densities were quantified for different branch orders. Pyr neurons had only one apical branch segment that was then divided into higher-order branches. The number and length of 2° and higher-order branches analyzed could vary across cells, depending upon cell anatomy and image acquisition. For dendritic puncta density averages, values for the Pyr 1° apical dendrite were not included, because this compartment showed a significantly higher density and appeared to be a distinct compartment of the neuron that may be contiguous with the somatic compartment.
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Automated input assignment

Presynaptic neurite reconstructions were created using automatic background subtraction thresholding of the presynaptic (PV, SST, or VIP) YFP channel, a split-touching objects diameter threshold of 1 μm, and 6 voxel minimum area settings in Imaris. For confocal stacks where presynaptic neurite YFP signal exhibited z-axis related signal drop-off, neurite reconstructions using automatic settings were generated separately for superficial and deeper optical sections of the stack. In such cases, both sets of presynaptic neurite reconstructions were visually examined for comparable density and size profiles.

Puncta spots were assigned to a specific presynaptic input using a distance threshold of 0.15 μm from spot centroid to the presynaptic neurite 3D-reconstructions edge. Methods using pre- and postsynaptic neurite colocalization may be confounded by false positives (where neurites are near the soma but do not synapse onto it) and false negatives (where a given neurite is associated with two or more postsynaptic sites that are conflated into a single crossing point). Indeed, in many cases, neurites made extended contacts with the soma surface that might include a single or multiple postsynaptic sites. We found that using postsynaptic puncta to differentiate multiple synapses along an extended region of presynaptic neurites enabled a more accurate estimate for the number of putative synaptic contacts (Figure 6).

Because distance parameters used to identify convergent signals could be digitally adjusted, we explored this space to establish a maximum distance for input detection of 0.15 μm. This was below the diffraction limit for our confocal images. As expected, use of larger distance thresholds for detecting inputs resulted in a substantial increase in the number of assigned puncta. These values were out of range for other published values for inhibitory synapse density and provided confirmation that smaller distance thresholds were more stringent and likely to be more accurate.

Statistical Analysis
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All reported values are mean ± SEM, unless otherwise stated. Dendritic puncta density is mean spot density per linear dendritic segment for a given cell. Soma density is total somatic spot count divided by soma surface area. Density distributions were tested for normality both within and across cells using the Shapiro-Wilk normality test. Within cells, all but two Pyr cells had normally distributed dendritic puncta densities. For these two cells, median dendritic puncta density was used to represent these cell’s average dendritic puncta densities. Mean dendritic puncta density was used for all other cells. One-Way Repeated Measures (RM) ANOVA was used to detect dendritic segment-level dependent puncta density (p < 0.05). Pearson’s correlation was employed to test the relationship between Pyr soma surface area and synapse density. Two-Way RM ANOVA was used to detect differences in the proportion of input-assigned synapses across Pyr compartments and input-types (p < 0.05). Post-hoc Tukey’s multiple comparison testing was performed to identify significant group mean differences for anatomical data. For physiological data, unpaired Student’s T-test was used to identify significant differences in mean mEPSC and mIPSC amplitude and frequency, and Kolmogorov-Smirnov test was used to test for differences in amplitude distributions (p < 0.05). All analyses were performed using Origin 2017 statistical software (OriginLab, Northampton, MA).

Results

FAPpost targeting to postsynaptic sites

Neuroligins are ubiquitously expressed at postsynaptic sites (Bemben et al., 2015). We took advantage of the pan-synaptic localization of a previously characterized NL-1 based tether (post-mGRASP; (Druckmann et al., 2014; Kim et al., 2011; Kwon et al., 2018) to direct an extracellular fluorophore to postsynaptic sites. Because the trans-synaptic protein-protein interactions involved in GRASP and other protein complementation methods are irreversible and may be linked to synaptic stabilization (Scheiffele et al., 2000; Tsetsenis et al., 2014), we replaced post-mGRASP’s extracellular GFP fragment with an intact FAP or YFP and packaged the modified construct into
recombinant AAV virus for expression under the control of the human synapsin promoter (Figure 1A,B). Virus was injected into mouse primary somatosensory (barrel) cortex for sparse neuronal labeling (Figure 1C). Transduced cells were identified in fixed tissue specimens using both dTom and FAP expression after MG dye labeling, without further signal amplification (Figure 1D-G).

*In vivo* 2-photon (2P) imaging of YFPpost-transduced dendrites from L2 Pyr neurons in mouse S1 revealed that punctate YFP signal was associated with both dendritic shafts and spines (Figure 1H-J). Because FAPpost fluorescence required the addition of MG fluorogen, and *in vivo* imaging was carried out using a cranial window with a glass coverslip after several days of recovery, it was not straightforward to image FAPpost expression *in vivo*. Our results indicate that YFPpost is bright enough for detection of puncta in living tissue. Overall, we find that NL-1 tethered fluorophores can be detected in both fixed and living brain tissue without signal amplification, with punctate expression that localizes to sites of synaptic input.

**Synapse localization without functional disruption**

Overexpression of other genetically-encoded synaptic proteins has been associated with elevated synapse density and abnormal electrophysiological properties. For example, increased anatomical synapse density and mEPSC frequency has been observed with overexpression of GFP-tagged PSD-95, gephyrin, or intact NL-1 (Chubykin et al., 2005; El-Husseini et al., 2000; Gross et al., 2013; Prange et al., 2004). Trans-synaptic interactions for split protein indicators have been shown to increase binding affinities for the tagged proteins, and irreversible GFP-reconstitution can perturb synapse stability and organization (Tsetsenis et al., 2014; Yamagata and Sanes, 2012).

Electrophysiological recordings can be a sensitive way to survey alterations in synaptic function, independent of anatomical quantitation from fluorescence images. To test whether FAPpost expression was associated with altered mEPSC and mIPSC properties, adjacent untransfected and dTom+, FAPpost transfected cells were targeted for whole-cell recordings (Figure 2).
FAPpost expression did not alter mean mEPSC frequency or amplitude (Figure 2B; frequency untransfected 2.3±0.5 Hz vs FAPpost 1.9±0.4 Hz; amplitude untransfected 12.5±1.0 pA versus FAPpost 12.8±0.7 pA). Furthermore, mean mIPSC frequency and amplitude were not significantly different (Figure 2E; frequency untransfected 1.8±0.5 Hz versus FAPpost 2.3±0.4 Hz; amplitude untransfected 11.5±0.9 pA vs FAPpost 10.8±0.6 pA), although a small reduction in the frequency distribution of mIPSCs was observed (Figure 2F). Thus, expression of postsynaptic fluorophores using NL-1 targeting sequences can be a non-invasive way to identify and quantitate synaptic distributions without altering synaptic function.

To test whether FAPpost synaptic labeling was detecting inhibitory synaptic contacts onto a cell, we evaluated the alignment of FAPpost signal with immunohistochemical detection of a ubiquitous presynaptic marker, bassoon (Richter et al., 1999). We focused on FAPpost labeling at the soma, since synapses here were easily detected in confocal cross-sections. Bassoon immunohistochemistry was carried out in PV-Cre x Ai3 tissue (where PV neurites were labeled with YFP), and bassoon alignment to FAPpost-expressing Pyr neurons in L2 was assessed (Figure 3A-E). More than 90% of bassoon puncta aligned with FAPpost puncta (Figure 3F), where only 7±10% (mean±SD) of bassoon+ PV lacked FAPpost signal. In 3/5 cells analyzed, we observed that all PV terminals showed both bassoon and FAPpost. This suggests that FAPpost labels that vast majority of synaptic contacts from PV neurons. FAPpost puncta showed a slightly lower rate of alignment with presynaptic bassoon (84±10% of detected FAPpost puncta could be aligned to a bassoon puncta; Figure 3G). For FAPpost puncta that did not show bassoon labeling, 12±6% were aligned to PV terminals – and were thus likely to be bona fide synaptic contacts. We attribute FAPpost and PV-aligned but bassoon immunonegative terminals to either true bassoon-negative release sites (Dondzillo et al., 2010) or incomplete labeling with the bassoon antibody, either from poor antibody penetration or due to small bassoon puncta that were not detectable given our labeling and imaging conditions. More than 70% of identified PV terminals also showed FAPpost expression (Figure 3H).
It is likely that some putative PV terminals apposed to the soma were not actual release sites, since the overwhelming majority (95%) of PV+ terminals aligned with FAPpost also showed presynaptic bassoon signal.

Since our analysis focused on PV inputs at the soma, these data provide evidence that FAPpost effectively labels at least one class of inhibitory synapses. Because we did not evaluate the presence of FAPpost at all synapse types defined by distinct pre- and postsynaptic cell types, we cannot be assured that it is equally distributed for all potential synaptic contacts.

We also carried out bassoon immuno-colocalization for FAPpost puncta along spiny dendrites from putative Pyr neurons. Analysis of 11 dendritic segments showed that >90% of FAPpost puncta were associated with bassoon (Figure 3I-M). Because synapses are densely distributed across a volume of brain tissue, and there are many synapses near a labeled segment that belong to an unlabeled pair of neurons, it was not possible to determine false negative rates (i.e. bassoon but not FAPpost for a given dendritic segment). Some FAPpost puncta had no detectable bassoon associated signal. This may result from either an inability to detect bassoon (low fluorescence signal or subthreshold levels of bassoon) or from a bona fide false positive, perhaps due to FAPpost signal that may not be synaptically localized.

High-throughput synapse quantitation

To facilitate high-throughput quantitative fluorescence analysis of synapses in neurons from brain tissue, we applied an efficient and scalable analysis pipeline with automated synapse detection and assignment. Sparse viral transduction of FAPpost in Pyr neurons from primary somatosensory (barrel) cortex revealed Pyr neurons decorated with bright, FAP puncta across the cell surface (Figure 4A). Using Imaris image analysis software, neural surfaces were rendered and puncta assigned to an individual neuron for quantitative analysis. Because dendritic spines were not always visible from the dTom fill, FAPpost puncta that were 1μm from the parent dendrite were digitally assigned to a given...
Reconstructions of fixed specimens yielded 200-1000 μm of continuous dendritic segment per neuron for analysis. Overall, FAPpost puncta densities across L2 Pyr dendrites, $2.3 \pm 0.1$ puncta/μm (excluding the 1° apical dendrite; see Methods) were similar to previous estimates of synapse density (Gulyas et al., 1999; Hersch and White, 1981; Holtmaat et al., 2005; Kasthuri et al., 2015; Villa et al., 2016a), supporting this high-throughput analytical approach. We also examined puncta densities in L2 Pyr neurons from the Emx1-Cre transgenic mouse strain using a Cre-dependent YFPpost construct (Figure 4-2). On average, puncta densities were elevated for L2 Pyr neurons in this strain (2.9±0.1 puncta/μm), consistent with the elevated spontaneous activity and enhanced seizure susceptibility that has been observed in this strain (Kim et al., 2013; Steinmetz et al., 2017).

We observed substantial heterogeneity in detected puncta density across individual L2 Pyr neurons, with close to four-fold variance across cells (1.0-3.8 puncta/μm), and nearly 10-fold variance (0.7 to 5.9 puncta/μm) across different dendritic branches (Figure 4G). We found no relationship between mean puncta density and total dendritic length analyzed for a given neuron. Variability in overall puncta density for any cell type could not generally be explained by sex, age, days post-infection, or animal-to-animal differences as neurons with a range of puncta densities could be found in the same animal (Table 1). It is likely that the number of viral particles infecting individual neurons was not uniform, even in cells analyzed from the same animal, and may be an additional source of heterogeneity in analysis of puncta densities; however, we found no significant relationship between puncta intensity and puncta density across analyzed neurons. Dendritic puncta density for a given cell was correlated with the soma density ($R^2=0.37$). Variability in observed puncta density for Pyr neurons is consistent with anatomical and electrophysiological response variability that has been described for this group of (Pronneke et al., 2015; Tasic et al., 2016; Tyler et
al., 2015; van Aerde and Feldmeyer, 2015; Yamashita et al., 2013; Yassin et al., 2010), and may reflect both developmental and molecular heterogeneity of neocortical Pyr neurons. Although we did not systematically evaluate FAPpost properties in inhibitory neurons, we observed punctate fluorescence in PV, SST, and VIP neurons that expressed this postsynaptic label. Thus, this tool may be useful for quantitative synapse analysis in multiple cell types. Quantitative analysis across different dendritic compartments revealed that the primary (1°) apical dendrite, a short region of dendrite that emerges from the soma of Pyr neurons, showed dense FAPpost puncta (Figure 4I). The high density of putative synapses in this compartment has not been well-described, in part because prior analyses have typically used dendritic spines as a proxy for synapses and this region is characteristically smooth. Mean puncta density on higher-order dendritic branches was similar across segments.

Presynaptic input assignment using fluorescence-based colocalization

Transsynaptic molecular complementation for synapse detection requires both pre- and postsynaptic transgene expression and may introduce unwanted effects on synapse function. In addition, because presynaptic input labels are not typically saturated using virally-introduced transgenes, complete and quantitative comparisons of input densities across cells and conditions are difficult. Here we assessed whether dendritic FAPpost could be used for identification of putative synaptic contacts where presynaptic neurites are fully labeled using Cre-dependent YFP expression in transgenic mice (Hippenmeyer et al., 2005; Taniguchi et al., 2011). The far-red emission of MG-binding FAPs can be easily multiplexed with other commonly-used fluorophores for detection of adjacent pre- and postsynaptic signals. Although many other studies have used the convergence of pre-and postsynaptic histochemical or fluorescence signal (Kubota et al., 2015; Schoonover et al., 2014) the introduction of a third feature that marks putative synapse location should improve the accuracy of input-specific synapse assignment in genetically-selectable, sparsely-labeled target cells.
Initially we focused on the primary apical dendrite of L2 Pyr neurons, where FAPpost puncta were clearly demarcated and densely distributed. Manual inspection of a confocal image series shows YFP-labeled PV neurites associated with the dendrite in close proximity to FAPpost puncta (Figure 5A). Digital analysis of this 3-dimensional segment for both puncta detection and neurite surface rendering enables a distance-based criterion for assigning specific puncta to PV inputs (Figure 5B-E).

A limitation of this fluorescence-based approach – for both us and in previous studies – is that diffraction–limited images cannot perfectly differentiate between apparent and true synaptic contacts. However, use of consistent analysis parameters across specimens may be sufficient to detect condition-specific changes in input organization.

How does inclusion of synaptic marker improve the detection of putative synapses? We compared the number of detected contacts using only fluorescence labeling of presynaptic neurites and the postsynaptic cell, or using these two features plus the presence of a FAPpost puncta at a contact site. We hypothesized that the number of putative synaptic contacts would be reduced when a third feature was required for synapse detection, and thus this method might offer improvements upon prior quantitative approaches. Analysis focused on YFP-labeled inputs to Pyr soma, where data from prior EM and light-microscopy analyses could confirm our analysis (Di Cristo et al., 2004; Hill et al., 2012; Kubota et al., 2016; Kubota et al., 2015; Melchitzky and Lewis, 2008; Tamas et al., 2000; Zhou et al., 2017).

Using only neurite-associations with the postsynaptic soma – a method that has been frequently used to estimate PV cell innervation (see for example (Di Cristo et al., 2004; Feldmeyer et al., 2006; Hill et al., 2012)) – we estimated the number of putative somatic synapses for individual Pyr neurons. We then compared these values for the same cell with additional requirement of a FAPpost puncta in between the neurite and the postsynaptic soma, using a neurite-to-puncta distance detection threshold of 0.15μm (Figure 6). The use of three features (presynaptic neurite, postsynaptic puncta, and postsynaptic neuron) to quantify input density reduced the number of putative contacts, or false...
positives, that likely result from non-synaptic neurite juxtaposition. In addition, we sometimes
observed neurite apposition at the soma that was associated with multiple underlying FAPpost
puncta, suggesting that prior methods using only pre- and postsynaptic proximity might have
underestimated actual synapse number. Overall, this analysis enabled us to obtain quantitative
information about synapse number using fluorescence imaging data more accurately than would be
provided by only pre- and postsynaptic neurite apposition.

We used this quantitative data to compare the density of inhibitory inputs to the soma across
three classes of inhibitory neurons. FAPpost puncta at the soma were more than 4-fold more likely to
be aligned with PV than SST neurites (mean±SD, somatic PV-assigned puncta 66±30, n=9 Pyr
neurons; versus somatic SST-assigned puncta 15±8, n=9 Pyr neurons, Figure 6). Analysis of VIP-
associated inputs revealed a small number of colocalized post-synaptic puncta at the soma (somatic
VIP-assigned puncta 11±16, <5% of total somatic puncta; n=9 Pyr neurons). Taken together,
approximately one-third of somatic puncta could be assigned to either PV, SST, or VIP inputs; the
stringency of our input-detection parameters likely underestimates the number of contacts,
particularly for PV neurons. Our findings are consistent with prior reports showing that the majority
of somatic inputs arise from PV neurons, with a minority of other inhibitory inputs (Di Cristo et al.,
2004; Hill et al., 2012; Kubota et al., 2016; Melchitzky and Lewis, 2008; Micheva and Beaulieu,
1995), and show that synapse identification improves the accuracy of quantitative input analysis.

Somatic and dendritic inhibition is dominated by PV input

It is commonly held that PV inputs preferentially target the soma and SST inputs, the dendrites
(particularly in L1; (Chen et al., 2015; Dienel and Lewis, 2018; Pi et al., 2013)). However,
quantitative evidence for this is lacking and indeed recent reports suggest that PV inputs may be
broadly arrayed across the dendritic arbor (Kubota et al., 2015). We compared the distribution of
PV, SST and VIP inputs across the soma and along Pyr dendrites, including 4° branches that could
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extend >140μm from the soma center (Figure 7, with gallery of PV-assigned, SST-assigned, and VIP-assigned inputs on individual L2 Pyr neurons in Figure 7-1, 7-2, and 7-3). Because soma size could vary more than two-fold between neurons (complicating measures of density), we compared the percent of total puncta that could be assigned to PV, SST, or VIP inputs for individual Pyr soma. Somatic puncta were dominated by PV inputs, where on average 25% of FAPpost puncta could be assigned to adjacent PV neurites but only 5% of FAPpost puncta could be assigned to SST inputs. In general, VIP inputs were rarely observed on L2 Pyr soma (Figure 7C,F).

Quantitative input assignment revealed that PV inputs were frequently observed along all dendrites where their distribution only modestly declined at higher branch orders (up to 4° branches; Figure 7F,H). On average, dendritic SST inputs were less abundant than PV inputs, using either density measurements (mean density dendritic SST-assigned puncta 0.20±0.03/μm versus PV-assigned puncta 0.38±0.07/μm, excluding the 1° apical dendrite for both) or relative proportion of assigned puncta (Figure 7G). Even in higher-order (3° and 4°) apical dendrites, SST inputs were not more numerous than PV inputs (Figure 7H). It remains possible that the L1 apical tuft of L2 Pyr neurons (that was not included in our analysis, due to sectioning artifacts) may contain dense SST inputs. Overall, quantitative input analysis shows that both PV and SST inputs are broadly distributed across the dendrites of Pyr neurons within L2/3.

Interestingly, we observed a pronounced concentration of PV inputs at the synapse-dense 1° apical dendrite as it emerged from the soma in L2 Pyr neurons (Figure 7A,B,H), with a significant 6-fold greater density than for SST inputs. These data suggest that the 1° apical compartment might be an extension of the soma with respect to PV presynaptic targeting and synaptic integration properties. The prominent absence of SST inputs at the somatic and 1° apical dendrite suggests that SST neurons may selectively avoid these PV-input-enriched perisomatic compartments (Figure 7G, H). Cortical wiring diagrams showing SST input are frequently schematized to indicate the apical dendrite or L1 as the primary site of synaptic input (Chen et al., 2015; Dienel and Lewis, 2018; Pi et al., 2013).
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data indicate that SST inputs are detectable across the dendritic arbor and may not be restricted to this layer.

VIP inputs to Pyr neurons showed a slightly higher density for the 1° apical versus other dendrites, although the absolute number of synapses was very low. Overall, VIP input density was 10-fold lower than PV inputs to the 1° apical dendrite, a difference that was highly significant (Figure 7G). For higher-order dendrites, VIP input density was significantly lower than either PV and SST inputs (mean density, dendritic VIP-assigned puncta excluding the 1° apical dendrite 0.07±0.01/μm). These differences were also reflected in the relative proportion, in addition to the density, of assigned FAPpost puncta. For example, at higher-order apical dendrites the proportion of total SST-assigned puncta (10.5±1.9% of total inputs) was slightly lower than the proportion of PV-assigned puncta (16.2±1.9% of total inputs), but significantly greater than VIP inputs (1.4±1.9%).

Because there are a small fraction of PV-Cre expressing Pyr neurons in deep layers, it is possible that some of the detected PV inputs may arise from PV-expressing Pyr neurons. However, PV-expressing Pyr neurons are not observed in L2, and it is likely that the majority of PV inputs arise from intralaminar inputs (Fino and Yuste, 2011). These findings are consistent with meticulous neuroanatomical reconstructions of synaptically-connected pairs showing that PV synapses can be observed across the dendritic arbor of neocortical Pyr neurons (Kubota et al., 2015). In addition, our data indicate that SST inputs are common across the dendritic arbor within L2. These data suggest revisions to previous cortical wiring diagrams that show SST inputs exclusively at the apical tuft and PV inputs exclusively at the soma (Chen et al., 2015; Dienel and Lewis, 2018; Pi et al., 2013).

Discussion

Synapses are a critical determinant of neural function, and their individual and collective properties can provide insight into how brain circuits are organized and changed by experience. Electrophysiological measurements of mEPSC and mIPSCs have been widely used to assess circuit-
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level adaptations in synaptic function, but typically sample only a small subset of inputs onto a neuron close to the recording electrode due to electrical filtering of small and distant signals. In contrast, anatomical methods offer a highly quantitative, compartment-specific and anatomically broad view of how synapses and cell-type specific inputs are distributed onto a neuron.

A fluorescence-based, molecular genetic platform for synaptic detection and quantitation has multiple advantages for high-throughput and scalable analysis. First, the brightness of FAP/YFPpost synaptic tags enable direct visualization of synapses in both live and fixed tissue without amplification, making them accessible tools for broad scale use. Second, synaptically-targeted fluorophores can be sparsely expressed in brain tissue, not just cultured neurons, to reveal properties of synaptic and input organization in a complex neural circuit. Third, fluorescence imaging enables use of multiple, spectrally distinct channels for cell-type-selective identification of axonal inputs and specific molecules that can differentiate synapses. Fourth, volumetric data collection is rapid and requires only a confocal microscope, and images can be used for high-throughput, automated analysis. Overall, quantitative and high-throughput synapse detection with FAP/YFPpost will facilitate cell-type specific characterization of synapses and connectivity changes across multiple animals and diverse experimental conditions.

Synaptic quantitation without perturbation

Experimental evidence indicates that FAPpost labels both excitatory and inhibitory synapses. FAPpost puncta are localized to soma and dendritic shafts, preferred targets for inhibitory synapses as well as on dendritic spines where excitatory synapses lie. FAPpost convergence with presynaptic inputs from confirmed GABAergic neuron subtypes, specifically PV, SST, and VIP neurons, indicates association with inhibitory inputs. The FAPpost labeling of both excitatory and inhibitory synapses allows comprehensive analysis of synapse distribution from a single postsynaptic marker.

Reagents that separately enable visualization of excitatory and inhibitory synapses will also be useful.
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tools for fluorescence-based quantitative imaging (Chen et al., 2015; Gross et al., 2013), if expression levels are high enough for reliable synapse detection.

Although it has been proposed that NL-1 is specifically targeted to excitatory synapses (Song et al., 1999), NL-1 contains a conserved binding motif for the GABAergic receptor scaffolding molecule gephyrin and NL-1-based synapse labeling constructs are sometimes observed at inhibitory synapses (Bemben et al., 2015; Kwon et al., 2018; Tsetsenis et al., 2014). Neurexin 1β-binding to the extracellular portion of NL-1 has been shown to enhance intracellular PSD-95 interactions (Giannone et al., 2013) and this region’s deletion in FAPpost may enable the broader distribution observed in transduced neurons. Importantly, prior studies have shown that the absence of the extracellular NL-1 region inhibits ectopic synapse formation (Chih et al., 2005), supporting the use of FAPpost as a non-invasive tag for synapse monitoring.

Does FAPpost expression alter synaptic function in vivo? This is a significant issue, as the electrophysiological effects of other fluorescence synapse detection reagents have not been well-investigated (Choi et al., 2018; Kim et al., 2011; Martell et al., 2016). Overexpression of tagged synaptic molecules leads to an increase in overall synapse number, using electrophysiological or anatomical measurements (El-Husseini et al., 2000; Gross et al., 2013). In such cases, quantitative analysis can be misleading, reflecting either a primary overexpression effect or a secondary effect of circuit-level adjustments to abnormal synaptic input. The absence of a clear electrophysiological phenotype for the FAPpost reagent suggests that overexpression of our fluorophore-tagged NL-1, in the absence of trans-synaptic interactions, may have a minimal effect on synaptic function.

Synapse detection accuracy

How does FAPpost synaptic quantitation compare to previous estimates of synaptic density and input organization? Synapse density has often been estimated indirectly from fluorescence images, using spines as a proxy for synapses. This is problematic, as spine detection will underestimate synapse number by excluding shaft synapses (typically inhibitory), dually-innervated spines (>10%
by some estimates (Chen et al., 2012), spines that lie within the imaging plane, and will also undercount faintly labeled, filamentous spines. Overall synapse densities for L2 Pyr neurons revealed an overall average of 2.8 synapses/μm dendrite, a density that is well within the range of prior estimates. For example, whole-cell EM reconstructions of CA1 hippocampal neurons have shown synapse densities of 0.7-7 synapses/μm, depending on location within the dendrite (Gulyas et al., 1999). Other studies analyzing spine (not synapse) density from L2 or L5 Pyr neurons in mouse S1 report between 0.4-5.1 spines/μm, where EM studies typically reveal greater spine densities (Holtmaat et al., 2005; Kasthuri et al., 2015; Villa et al., 2016a).

Fluorescence-based synaptic tags reduce multiple sources of error that can inaccurately assess total synapse number. For isolated neurons, automated puncta assignment to the parent dendrite removes the requirement that dendritic spines be visible for synapse detection, reducing false-negative rates. For automated assignment, this rate will always be non-zero as the distance limit set for puncta assignment will exclude puncta that lie on longer dendritic spines, which can extend >5μm in some cases (Kasthuri et al., 2015).

False-positive (non-synaptic puncta) errors are more difficult to estimate. Intracellular pools of the targeting construct may contribute. Although these were digitally excluded based upon distance to the plasma membrane, this process that may not be effective for thin dendritic segments. It is possible that non-synaptic, plasma-membrane FAPpost accumulation may sometimes occur. In addition, puncta from nearby neurons may have been inadvertently misassigned to an analyzed dendritic segment. While fluorescence-based genetic methods have advantages, they are subject to variations in expression levels, both of the labeling construct and of protein trafficking to different synapses (for example, that may have a lower NL-1 content or are more distant from the soma). It remains possible that not all synapses – for example, neuromodulatory or peptidergic inputs – were uniformly labeled using this methodology. The quantitative analysis pipeline established here...
Quantitative cell-type specific connectomics attempts to reconcile high-throughput analysis with variability in synapse structure, where speed and accuracy must be balanced.

Biological and non-biological variability

We observed marked within (10-20-fold) and across cell (2-4-fold) variability in FAP/YFPpost synapse density within and across L2 Pyr neurons. Variability in synapse density and its biological implications has not been well-explored. Most analyses have focused on complete reconstruction of a single neuron (Megías et al., 2001) or of a few dendritic segments (Kasthuri et al., 2015; Villa et al., 2016 and others). In one study that carried out a detailed analysis of multiple pyramidal cells’ apical dendrites, a wide range of synapse densities were observed (Hersch and White, 1981). Cells with higher synapse densities may represent “hub” cells that receive wide distribution of synaptic inputs, or more recurrent connections from the same presynaptic neuron(s). Notably, miniature postsynaptic current frequency data (both ours and others) shows a 10-fold range in values across pyramidal neurons. Mini frequencies are typically interpreted as reflecting the number of synaptic connections on a given postsynaptic cell. Considering the electrophysiological correlate of synapse number shows a similar range in values to anatomical correlates of synapses on pyramidal neurons, we may very well be capturing normal biological variability in synapse numbers.

Alternatively, observed variability may be a non-biological labeling artefact. To achieve complete synaptic labeling, expression of any synapse-tagging molecule must reach sufficient levels in an individual cell to label all synapses across its entire dendritic arbor. It is unlikely that this labeling occurs at the same time for all cells. One alternative explanation for the wide-range in synapse densities is that only a fraction of synapses were labeled in a particular cell. We attempted to control for this by only selecting well-labeled cells, but we cannot rule out this potential confound in the interpretation of our findings.

Volumetric imaging for high-throughput synaptic input assignment
A significant advance enabled by an all-fluorescence synaptic imaging platform is the automated assignment of cell-type specific synaptic inputs with a spectrally distinct fluorophore. The tricolor (FAPpost, dTom, and presynaptic YFP) association as a criterion for synapse detection substantially reduces the false positive rate compared to brightfield microscopy methods (Hill et al., 2012; Kubota et al., 2015; Schoonover et al., 2014). We took advantage of the complete labeling of molecularly-defined inhibitory neuron populations in Cre-driver transgenic mouse lines to examine the broad-scale distribution of FAPpost labeled synaptic inputs on L2 Pyr neurons that originated from three types of GABAergic neurons.

Our analysis revealed that PV inputs predominate at somatic locations, with approximately six times as many PV as SST inputs to the cell body. These data are consistent with reports of dense PV innervation of the soma (Kubota et al., 2016) and a small fraction of somatic SST inputs (<10%) (Hill et al., 2012), and further validate FAPpost labeling as a robust method for quantitative synapse assignment. Dendritic analysis of synapse organization identified the 1° apical dendrite of Pyr neurons as a site of particularly dense PV innervation. This aspiny region of the dendrite, particularly in neocortical Pyr neurons, has been poorly studied as prior imaging methods have not been able to reliably visualize synapses in this compartment. Importantly, whole-neuron EM reconstructions show that >90% of inputs to the apical dendrite of CA1 neurons are inhibitory (Bloss et al., 2016; Megías et al., 2001). The distinctive properties of the apical dendrite (Major et al., 2013) suggest that PV input to this region may serve as a critical filter for top-down modulation of Pyr neuron firing in the neocortex.

Quantitative analysis showed that PV neurons have more input to Pyr neuron dendrites than other neocortical inhibitory neurons. Indeed, even when excluding the densely PV-innervated 1° apical dendrite, mean dendritic input density was greater for PV than SST, and VIP inputs. Although this may be incongruous with the simplified model that contrasts soma-targeting PV and dendrite-targeting SST inputs (Chen et al., 2015; Higley, 2014; Lazarus and Huang, 2011; Pakan et al., 2016;
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Pi et al., 2013), prior experimental data are much less categorical than these schema suggest. For example, anatomical reconstructions from paired whole-cell recordings show that the majority of PV inputs to neocortical pyramidal neurons are located >50μm from the soma (Hill et al., 2012; Kubota et al., 2015) and abundant SST contacts can be detected at both proximal and distal dendrites (Di Cristo et al., 2004; Hill et al., 2012). It remains possible that very distal dendrites, particularly in L1, have a disproportionate association of SST inputs. Based on the density of synaptic inputs, our data indicate that PV-mediated synaptic input will be the predominant source of inhibition across the somatodendritic compartments of L2 Pyr neurons.

Conclusion

This analysis helps generate a framework for large-scale anatomical imaging to examine circuit- and brain-wide changes in synapse distribution in development, learning, and disease. Future efforts should leverage volumetric imaging in cleared or expanded tissue for complete and high-resolution capture of the entire dendritic apparatus, application of additional molecular markers to distinguish different synapse types, and will employ new presynaptic constructs for improved synaptic discrimination. A critical challenge of these future possibilities will be the digital capture and storage of large anatomical datasets for computational analysis.
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References


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Figure/Table/Extended Data Legends

Figure 1: Construct design and expression in mouse somatosensory (S1 barreelfield) cortex. (A) FAPpost and YFPpost construct design. Human synapsin promotor (hSyn) driving either a constitutively-expressed or Cre-dependent (Fl) FAPpost and dTom, separated by a 2A sequence for independent localization. (B) Fl-YFPpost construct. Cre-dependent YFPpost and dTom expression. (C) Virus injection coordinates. (D) Confocal image stack of L2 Pyr cell transfected with FAPpost. Scale = 10μm. (E) Optical section of FAPpost puncta on soma of cell in D. Scale = 2μm. (F) Zoom of F. Arrow-head marks cytoplasmic FAPpost accumulation. Scale = 1μm. (G) FAPpost labeled spiny dendrites. Scale = 1μm. (H) In vivo 2P imaging schematic. (I) Single-plane 2P image of L2 Pyr cells transfected with of YFPpost and dTom. Scale=30μm. (J) Single-plane 2P image of YFPpost-labeled spiny dendrite in L1. Scale as in D+G. See also Movie 1.

Figure 2. FAPpost synaptic localization does not alter mEPSC and mIPSC properties. (A) Example voltage-clamp traces from an untransfected (black) and neighboring FAPpost-expressing (red) L2/3 Pyr cell showing mEPSCs. (B) Comparison of mean mEPSC frequency (ANOVA\textsubscript{Frequency}: F\textsubscript{(1,16)}=0.2, p=0.6) and amplitude (ANOVA\textsubscript{Amplitude}: F\textsubscript{(1,16)}=0.1, p=0.8) indicate no difference. (C) Cumulative distribution histogram of mEPSC amplitudes (Kolmogorov-Smirnov Test, D=0.05, p=0.16). (D) Example voltage-clamp traces from an untransfected (black) and neighboring FAPpost-expressing (red) L2/3 Pyr cell showing mIPSCs. (E) Mean mIPSCs frequency (left) of untransfected and dTom cells were not significantly different (ANOVA\textsubscript{Frequency}: F\textsubscript{(1,18)}=0.6, p=0.4). Mean mIPSC amplitude (right) of untransfected and dTom cells were not significantly different (ANOVA\textsubscript{Amplitude}: F\textsubscript{(1,18)}=0.5, p=0.5). (F) Cumulative distribution histogram of mIPSC amplitudes shows a small but significant shift in dTom mIPSC amplitudes (Kolmogorov-Smirnov Test, D=0.15, p<0.0001). n=8-11 cells, N=7 animals.
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Figure 3. FAPpost puncta align with presynaptic bassoon. (A) Optical section of a confocal image used to assess bassoon immunofluorescence alignment with PV terminals on soma of FAPpost labeled cell visualized in Ai3xPV-Cre mouse. Quadruple channel overlay showing presynaptic PV terminal (YFP, cyan) colocalization with bassoon immunofluorescence (Alexa405; yellow) and alignment with FAPpost puncta (green) on dTom (red) filled soma. Scale, 1μm. (B-E) As in A, but each channel in isolation. White arrows indicate triple-channel alignment example puncta, orange arrow indicates quadruple-channel alignment example puncta. (F) Presynaptic bassoon puncta rate of alignment with FAPpost (F+) and/or colocalization with PV terminals (P+). Bars are mean±SD of individual soma alignment rates (dots; n=5 soma; puncta assessed, n=104). (G) FAPpost puncta rate of alignment with bassoon (B+) and/or PV terminals (P+; dots; n=5 soma; puncta assessed, n=92). (H) PV terminal rate of alignment with FAPpost (F+) and colocalization with bassoon (B+; dots; n=5 soma, terminals assessed, n=83). (I) Triple channel overlay showing presynaptic bassoon immunofluorescence (Alexa405; white) alignment with dendritic FAPpost. Scale, 1μm. (J-L) As in I, but each channel in isolation. White arrowhead indicates FAPpost puncta not aligned with bassoon. (M) FAPpost rate of alignment with bassoon (B+) along separate dendritic segments (dots; n=11 dendritic segments; puncta assessed, n=143).

Figure 4. Synapse quantitation for L2/3 Pyr neurons. (A) Confocal stack of example FAPpost Pyr neuron. (B) Zoom of spiny dendritic segment. (C) Schematic for dendritic puncta assignment (green, assigned puncta <1.0μm from shaft surface; light green, unassigned puncta). (D+E) 3D-rendering of the neuron and puncta assignment. Scale = 10 μm, 2 μm. (F) Schematic of Pyr branch orders analyzed. (G) Mean FAPpost puncta density for individual neurons (grey bars,±SEM) on 2°-4° apical and 1°-4° basal dendritic branches (black dots). (H) Somatic and dendritic puncta density are correlated (R²=0.37, p=0.0003). (I) Mean FAPpost synapse density across 1°-4° Pyr branches (bar±SEM). (right) Individual cell values, plotted as connected lines. RM
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ANOVAPyr: $F(7,126)=19, \ p<0.0001$. All data shown (n=29 cells, N=12 animals), statistical comparisons performed on balanced data (n=19 cells, N=10 animals). See Extended Data Figure 4-1 for puncta detection using YFPpost and Extended Data Figure 4-2 for Imaris analysis workflow.

Figure 5. FAPpost puncta on the primary apical dendrite align with presynaptic PV neurites. (A) Six serial optical sections of a Pyr primary apical dendrite labeled with FAPpost (green) and dTom (red). (Top row) Fluorescence aligned with presynaptic PV (YFP; cyan). (Bottom row) FAPpost and dTom fluorescence alone. (B) Flattened stack of the region in A, showing PV(YFP) and dTom. (C) Rendering of B. (D) As in B, but for FAPpost and dTom. (E) Rendering of PV-assigned FAPpost puncta (large red balls) and unassigned (small green balls) puncta. Scale=1μm.

Figure 6. FAPpost detection improves estimates of input association. (A) Diagram illustrating distance parameters used for FAPpost puncta assignment to soma-surface (left), soma-puncta association with presynaptic YFP-expressing neurites (middle), and presynaptic neurite associations with soma-surface (right). (B) Comparison of the number of presynaptic neurite to soma-surface and soma-puncta associations. Connected lines = individual cell values. More PV neurite to PYR soma-surface (115±16) than soma-puncta contacts were detected (66±30; Paired T-Test, $t=3.3, \ p=0.01$). More SST neurite to soma-surface (69±10) than soma-puncta contacts were detected (15±8; Paired T-Test, $t=6.7, \ p=0.0001$). Number of VIP neurite to soma-surface (16±4) and soma-puncta contacts were similar (11±5; Paired T-Test, $t=1.1, \ p=0.3$). (C) Contact overestimation depicts the difference between presynaptic neurite to soma-surface and soma-puncta associations for presynaptic PV neurites (+49±15), SST neurites (+54±8), and VIP neurites (+5±4). Negative values occurred when multiple puncta associated with a single presynaptic terminal. (D) Error rate for presynaptic neurite-to-soma associations versus soma puncta associations for PV (115±56%), SST (394±70%), and VIP inputs (280±162%). Across PYR cells, the percentage overestimation did not vary by input cell type (ANOVA_{CellType}: $F(2,24)=1.7, \ p=0.2$). PV input: n=9 cells, N=4 animals; SST
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input: n=9 cells, N=4 animals; and VIP input: n=9 cells, N=3 animals. (E) Optical section of a
confocal image used to assess bassoon immunofluorescence alignment with PV terminals on soma of
FAPpost labeled cell visualized in Ai3xPV-Cre mouse. Scale, 2μm. (F) Boxed region from A,
quadruple channel overlay showing presynaptic PV terminal (YFP, cyan) colocalization with bassoon
immunofluorescence (Alexa405; yellow) and alignment with FAPpost puncta (green) on dTom (red)
filled soma. Scale, 0.5μm. (G-I) Same as B, but for PV, bassoon, and FAPpost visualized with dTom.

Figure 7. The distribution of PV, SST, and VIP inputs across L2 Pyr neurons. (A) PV-
input assigned synapses for an example L2 Pyr neuron. Small light-green spheres are un-assigned
FAPpost puncta; large colored spheres are input-assigned FAPpost puncta. See Figure 7-1 for images
of all input-analyzed Pyr neurons. (B) Mean density of PV-assigned FAPpost contacts across
dendritic branch orders. (left) Bar is mean±SEM of all cells; (right) Individual cell values, plotted as connected lines. All data shown, statistical comparisons performed on balanced data. PV-assigned puncta density was greater for the 1° apical dendrite. RM ANOVA_PV-Input: $F(7,28)=6.7, p=0.002; n=5$ cells, N=3 animals. *Tukey post-hoc pairwise comparison test, $p<0.05$. (C) As in A but for SST. See Figure 7-2 for images of all input-analyzed Pyr neurons. (D) SST-assigned puncta density was not statistically significantly different across branch orders. RM ANOVA_SST-Input: $F(7,28)=0.63, p=0.7$; n=5 cells, N=3 animals. (E) As in A but for VIP. Scale=20 μm. See Figure 7-3 for images of all input-analyzed Pyr neurons. (F) VIP-assigned puncta density was greater for the 1° apical dendrite. RM ANOVA_VIP-Input: $F(7,42)=3.8, p=0.003; n=7$ cells, N=3 animals. *Tukey post-hoc pairwise comparison test, $p<0.05$. (G) Inhibitory innervation of Pyr neurons, expressed as a percent of the total number of detected synapses, for each input source. All dendritic compartments pooled for somatic and dendritic comparison. Two-Way RM ANOVA_input: $F(2,24)=45, p<0.001$. All * show Tukey post-hoc pairwise comparison test, $p<0.05$. (H) Pie-chart showing average proportion of input-assigned
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FAPpost contacts versus total detected synapses, binned as perisomatic (soma and 1° apical) or higher-order dendritic compartments (apical 3°+4° and basal 3°+4°). A greater proportion of PV-inputs were found on soma and 1° apical dendrite than for SST- or VIP-inputs. At higher-order apical branches, the proportion of SST-input (10.5 ± 1.9%) was similar to PV-input (16.2 ± 1.9%), but significantly greater than VIP-inputs (1.4 ± 1.9%). For higher-order basal branches, all input sources were significantly different (PV = 17.3 ± 1.4%, n=7 cells, N=4 animals; SST=10.2±1.4%, n=7 cells, N=4 animals; VIP = 2.3±1.4%, n=7 cells, N=2 animals). Two-Way RM ANOVA: F(2,18)= 47.3, p<0.0001.

Movie 1. L2/3 Pyr neuron labeled with dTom and FAPpost. Confocal image stack of an isolated L2/3 neuron with pyramidal morphology showing punctate FAPpost (pseudocolored green) along the soma and dendritic arbor.

Table 1. Experimental metadata.

Figure 4-1. YFPpost fluorescent puncta quantitation in L2/3 Pyr cell dendrites. (A) Sparse YFPpost expression across the cortical column. Scale=50 μm. (B) L2/3 Pyr expressing YFPpost. (C) YFPpost fluorescent puncta on a dendritic shaft and spines (zoom from box in B). (D) Schematic for dendritic puncta assignment (blue, assigned puncta ≤1.0μm from shaft surface; light blue, unassigned puncta). (E) 3D-rendering of Pyr neuron (red) with assigned puncta. Scale=20μm. (F) Dendrite from C with assigned puncta. Scale=2μm. (G) Mean YFPpost puncta density for individual neurons (grey bars,+SEM) on apical and basal dendritic branches (black dots). n=21 cells, N=4 animals. See also Figure 4-2 and Table 1.

Figure 4-2. Fluorescent puncta detection method using Imaris. (A) Image files imported into Imaris. Scale=40μm. (B) Raw synaptic fluorescent signal. (C) Gain-adjusted view of synaptic fluorescent signal to visualize dim YFP signal. (D) White mask of synaptic fluorescence differentiates signal from background. (E) Puncta creation parameters: 0.5 μm estimated diameter, larger than 3 voxel size (grey pixels). (F) 3D-renderings of all fluorescent puncta (yellow). (G)
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Puncta within 0.5 μm from cell surface (edge-to-edge; blue). (H) Isolated cell-associated puncta (blue). (I+J) Cytoplasmic puncta (≤ 0.5 μm from cell surface; yellow). Scale=5μm. (K+L) Isolated cell-surface puncta. (M) Raw synaptic fluorescence used for Imaris spot detection. (N) Local fluorescence intensity maxima identified using automatic detection parameters (yellow spots). (O) 3D-rendering of puncta centers (from L) as spots. (P+Q) Alignment of puncta 3D-renderings and spots. Scale=1μm. (R) Alignment of spots and signal enhanced fluorescence. (S+T) Alignment of spots and raw fluorescence. Scale=5μm. (U) Workflow summary.

**Figure 7-1. PV-assigned FAPpost puncta distribution across Pyr cells.** Small light-green spheres are un-assigned FAPpost puncta. Large colored spheres are input-assigned FAPpost puncta. Scale=15μm.

**Figure 7-2. SST-assigned FAPpost puncta distribution across Pyr cells.** Small light-green spheres are un-assigned FAPpost puncta. Large colored spheres are input-assigned FAPpost puncta. Scale=15μm.

**Figure 7-3. VIP-assigned FAPpost puncta distribution across Pyr cells.** Small light-green spheres are un-assigned FAPpost puncta. Large colored spheres are input-assigned FAPpost puncta. Scale=15μm.
Fig5
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Table 1. Experimental metadata