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Synaptic organization of cortico-cortical communication in primates

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Abstract

In cortical circuitry synaptic communication across areas is based on two types of axon terminals, small and large, with modulatory and driving roles, respectively. In contrast, it is not known whether similar synaptic specializations exist for intra-areal projections. Using anterograde tracing and three-dimensional reconstruction by electron microscopy (3D-EM) we asked if large boutons form synapses in the circuit of somatosensory cortical areas 3b and 1. In contrast to observations in macaque visual cortex, light microscopy showed both small and large boutons not only in inter-areal pathways, but also in long-distance intrinsic connections. 3D-EM showed that correlation of surface and volume provides a powerful tool for classifying cortical endings. Principal component analysis supported this observation and highlighted the significance of the size of mitochondria as a distinguishing feature of bouton type. The larger mitochondrion and higher degree of perforated

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postsynaptic density associated with large rather than to small boutons support the driver-like function of large boutons. In contrast to bouton size and complexity, the size of the postsynaptic density appeared invariant across the bouton types. Comparative studies in human supported that size is a major distinguishing factor of bouton type in the cerebral cortex. In conclusion, the driver-like function of the large endings could facilitate fast dissemination of tactile information within the intrinsic and inter-areal circuitry of areas 3b and 1.

Keywords

tract tracing; electron microscopy; axon terminal; serial section reconstruction; multivariate analyses; *Saimiri sciureus*; human

Introduction

Communication between cortical areas and between cortex and thalamus is mediated via two major types of axon terminals, large ones (Class 1) with driver-like physiological function and small ones (Class 2) with modulatory function (Petrof and Sherman, 2013). Reciprocal connections between cortical areas form both small and large terminals (Anderson et al., 1998; Anderson and Martin, 2006, 2009; Covic and Sherman, 2011; Petrof et al, 2015). Anderson and Martin (2009) also provided morphological evidence that size and shape of the postsynaptic density (PSD) and mitochondrial content of axon terminals vary with bouton size, features which could play important roles in shaping the physiological properties of synapses (Pierce and Lewin, 1994). Indeed, presence of mitochondria, and large, complex, perforated, and/or multiple PSDs are associated with high synaptic efficacy and plasticity (Nikonenko et al., 2002; Vos et al., 2010; Holderith et al., 2012; Devine and Kittler, 2018). In spite of numerous studies describing differences in presynaptic characteristics including mitochondrial content, organization of PSD and vesicular number no universal quantitative criteria distinguishing Class 1 and Class 2 type endings have been determined (Germuska et al. 2006; Eyre et al., 2007; Hsu et al., 2017; Rodriguez-Moreno et al., 2018; Zikopoulos and Barbas, 2007; Wang and Barbas, 2018).

Another prevalent type of connectivity in cerebral cortex is intrinsic, intra-areal connectivity. Such connections have been implicated in functions such as surround suppression, neural synchronization, and shaping of receptive properties. However, the synaptic characteristics of intrinsic, intra-areal connections is less well understood. While the target selectivity of intrinsic synapses formed by local axon collaterals has been studied (Somogyi et al., 1998; Lewis et al., 2002; White et al., 2007), the ultrastructural characteristics have not been examined in depth at a quantitative level. Classification of synaptic boutons based on quantitative criteria may help reveal the distinct functions of cortico-cortical connectivity (DeFelipe, 2010).

In our previous tract tracing light microscopic studies on the reciprocally connected somatosensory cortical areas 3b and 1 of squirrel monkeys (Negyessy et al., 2013; Ashaber et al., 2014) we found a small fraction of boutons with relatively large size, which raised the question that communication in this circuitry is also mediated via Class 1 and Class 2 type endings. Little is known at the synaptic ultrastructural level about the connections and

organization of areas 3b and 1 (Sur et al., 1980; Shanks et al. 1985; Iwamura, 1998; Kaas, 2004; Pei et al 2010; Palfi et al., 2018). Here, following focal iontophoretic injections of the anterograde tracer BDA in areas 3b and 1, we analyze large and small terminal boutons with serial section electron microscopy to address two main questions. First off, this report focuses on examining whether large, driver-like synaptic boutons contribute to inter-areal as well as intrinsic connectivity within areas 3b and 1. The present findings show that long range intrinsic as well as inter-areal pathways form both types of boutons in the somatosensory cortex of squirrel monkey. The second goal was to determine which ultrastructural features can distinguish large driver-like boutons from small terminals. Our hypothesis was that the size of boutons is fundamentally determined by the size and number of organelles and structures including mitochondria, synaptic vesicles and active zone where molecular cascades of second messenger mechanisms are taking place within the boutons, and therefore could be useful variable in classifying cortical axon terminals. We used single variate and multivariate PCA (principal component analysis) to distinguish the power of different 3D ultrastructural variables to classify small and large terminals. PCA determines the correlated and non-correlated set of variables in the data, and is a favorable technique when there is a high possibility of correlation between the variables. Accordingly, in hippocampus using factor and cluster analysis and three dimensional (3D) reconstructions Eyre et al. (2007) failed to classify boutons of diverse inhibitory cell types due to correlations between the variables. Strong correlations were also found between different morphological variables of excitatory terminals of the cerebral cortex (Pierce and Lewin, 1994; Germuska et al. 2006; Hsu et al., 2017; Rodriguez-Moreno et al., 2018; Yakoubi et al., 2019a,b). We show that correlation of bouton surface and volume distinguishes Class 1 and Class 2-like cortical axon terminals and that PCA corroborates this observation and supports a role of mitochondrion in distinguishing between bouton types. To verify our approach and generalize our findings additional analyses of synaptic boutons of layers 4 and 5 of the human temporal cortex (Yakoubi et al., 2019a,b) further supported that size is a major determining factor of bouton type in the cortex. Preliminary findings of this study have been published in abstract form (Ashaber et al., 2016, 2019).

Materials and methods

Animal preparation, surgery and tracer injection

Animal care and surgeries were performed according to NIH (National Institutes of Health) regulations and approved IACUC (Institutional Animal Care and Use Committee) protocols. All procedures were in compliance with and approved by the Institutional Animal Care and Use Committee of Vanderbilt University. Three male and three female adult squirrel monkeys (*Saimiri sciureus* weighing 600–800 g, 2–9 years old) were used, which were subjects in our prior studies on the connectivity of the distal finger pad representations of area 3b and area 1 (Negyessy et al., 2013; Ashaber et al., 2014; Pálfi et al 2018). Here we provide a brief description of the experimental procedures (for details, see Négyessy et al. (2013) and Ashaber et al. (2014)).

Each animal was sedated (ketamine, 15 mg/kg, im), placed in a stereotaxic frame, mechanically ventilated with isoflurane anesthesia and hydrated with lactated ringers via

intravenous infusion. Analgesia during surgery was supplied by buprenorphine (0.01 mg/kg, im). Vital signs (blood oxygen saturation (SpO₂), heart rate, ECG (electrocardiogram), ET-CO₂, respiratory pattern, temperature) were monitored. After a craniotomy (centered at AP 6 mm and ML 15 mm) and durotomy, areas 3b and area 1 were located using the central sulcus and blood vessel landmarks. Following electrophysiological mapping of the hand and finger representations, intrinsic signal optical imaging (IOS) was used to identify the distal finger pad representations of fingers D2-D4 in areas 3b and 1. Injection of biotinylated dextran amine (BDA, 1:1 mixture of 10% 10K and 10% 3K, Molecular Probes, Inc. Eugene, OR, USA) in 0.01 M phosphate buffer (PB, pH 7.4) was then made into a distal finger pad representation in either area 3b or area 1 via iontophoresis (3 cases each). In all cases the core (300 µm in diameter) of the uptake zone of the BDA injection included the upper layers, while lower cortical layers were also included into the core in cases of area 1 injections (for more details see Table 1 in Négyessy et al. (2013) and Ashaber et al., (2014)). Upon recovery heat support was provided for the first 12 hrs with post-operative analgesia supplied by buprenorphine (0.01 mg/kg, im, twice a day) for 3 days. After a 10 to 20 day survival period animals were deeply anesthetized before perfusing transcardially with fixative consisted of 4% paraformaldehyde, 0.1 % glutaraldehyde and 0.2% picric acid in 0.1 M PB (pH 7.3). Brains were immediately removed, the region of interest was then cut from cortex, flattened parallel to the cortical surface and postfixated overnight in 4% paraformaldehyde.

Tissue Processing

Regularly spaced (at 130–160 µm except one case with 270 µm) series of 50 µm thick tangential sections were cut by vibratome sectioning (see Négyessy et al., 2013; Ashaber et al., 2014). The standard ABC protocol (Elite kit, Vector Laboratories, Inc. Burlingame, CA, USA) was used to visualize BDA labeling with nickel intensified diaminobenzidine (NiDAB) (Sigma-Aldrich, Budapest, Hungary) as the chromogen (for more details about the procedure see Négyessy et al. 2000; Négyessy et al., 2013). First the sections were cryoprotected (30% sucrose in PB) and tissue penetration enhanced by freezing-thawing. Unbound aldehydes were reduced by borohydride (1% NaBH₄ in PB, 30 min) and intrinsic peroxidase activity was blocked by 1% H₂O₂ in PB (30 min). Sections were then incubated in ABC (avidin biotin complex, 1:200 in PB, 0.1M, pH 7.4) overnight at 4°C. After the NiDAB reaction, sections were osmicated (1% OsO₄ (Electron Microscopy Sciences, Hatfield, PA, USA) in PB (pH 7.4) containing 5% sucrose for 60 minutes) and flat embedded in resin (Durcupan ACM, Sigma-Aldrich, Budapest, Hungary). Blocks containing selected BDA-labeled boutons were re-embedded and cut into a series of ultrathin sections (60 nm thick, silver color) that included the entire labeled bouton (but in most instances only included a partial portion of the surrounding postsynaptic dendritic structures) were cut with an ultra-microtome (Reichert). Ultrathin sections were collected onto Formvar coated grids, post-stained with lead citrate and examined with a JEOL JEM-1200EX electron microscope. Altogether 65 BDA-labeled boutons were cut and reconstructed in 3D.

Human samples used in Yakoubi et al. (2019a,b) were obtained from surgery of female and male patients (altogether 6 patients in Yakoubi et al. 2019a, and 7 patients in Yakoubi et al. 2019b) suffering from drug resistant temporal lobe epilepsy. Tissue blocks of temporo-

lateral and temporo-basal regions of the inferior temporal gyrus remote from the epileptogenic area were immersion-fixed in ice-cold solution containing 4% paraformaldehyde and 2.5% glutaraldehyde diluted in 0.1M PB (pH 7.4) for 24–48 h at 4°C. After fixation, 150–200 µm thick coronal section were cut with a vibratome and processed similarly as described above including post-fixation with OsO₄ and flat embedding in Durcupan ACM. Serial ultrathin sections were then cut from the region of interest, counterstained with uranyl acetate and lead citrate. Synaptic boutons were reconstructed from series of digital images by using the OpenCAR (Contour Alignment Reconstruction; for details see Sätzler et al. 2002) software tool.

Data analysis and morphometry

Light microscopy—On the series of vibratome sections, before dissecting and cutting for electron microscopy, all large BDA-labeled boutons were mapped under light microscopy to determine their intra-areal and inter-areal distribution with Neurolucida (MicroBrightField Europe, E.K. Magdeburg, Germany) using an Olympus research microscope equipped with a motorized stage (MultiControl 2000, Märzhäuser Wetzlar GmbH & Co. KG, Wetzlar, Germany). Within the injected area only long range intrinsic boutons were mapped as the dense NiDAB precipitation prevented mapping in the core and halo region of the injection (~500 µm in diameter). For mapping only BDA-labeled boutons larger than the size of the cursor, which was set to 1 µm, which is around the resolution limit of LM, were selected and plotted using 40x and 100x objective magnification. Although cortical axon terminals are usually small, equal or less than 1 µm in diameter, the diameter of large endings can exceed 1 µm (Anderson et al., 1998; Anderson and Martin, 2006, 2009; Négyessy et al., 2005; Covic and Sherman, 2011; Innocenti and Caminiti, 2017). The choice of the size limit of 1 µm allowed for the selection of large boutons to be mapped under light microscopy and helped to decrease the influence of the numerous Class 2 boutons on the analysis of the areal distribution.

The 3D structure of the large boutons were examined first by the Stereo Converter, a light microscopic system for 3D analysis (Hungarian patent no. P0800650; Stuber et al., 2012). This system enables the display of microscopic structures in the form of stereo-image pairs. An important feature of Stereo Converter is its ability to increase the optical magnification by a factor of 5 and importantly extend the depth of focus by twenty to thirty times of that of a typical lens objective. These optical features make the Stereo Converter suitable for the 3D analyses of light microscopic structures especially of thin neurites near the limit of resolution and supported the identification of the boutons, e.g. by excluding labeling artefacts, and thereby the selection for further EM examination.

Neurolucida-drawings of the distribution of boutons were pre-edited in Neurolucida Explorer (MicroBrightField Europe, E.K. Magdeburg, Germany). Photomicrographs of the labeled boutons were taken by a digital camera and edited (crop, rotate, minimally adjusting contrast and brightness when needed) with Adobe Photoshop (Adobe Systems, San Jose, CA). Borders between cortical areas were estimated based on electrophysiological mapping, receptive field characteristics, and optical imaging as described previously (Négyessy et al., 2013). Alignments between the functional maps, histological sections and serial

reconstructions of the BDA labeling were made in Neurolucida and Adobe Photoshop via fiduciary markers (e.g. blood vessels) (Negyessy et al., 2013). Figures were then composed in Adobe Photoshop.

Transmission electron microscopy—After mapping, tissue blocks including large (> 1 µm diameter under LM) BDA-labeled boutons selected by light microscopy, were cut serially for EM reconstructions. Our sample represented intrinsic and inter-areal large boutons from within and outside of terminal axon arborization patches (Négyessy et al, 2013; Ashaber et al, 2014 see also Lund et al. 1993) of upper and deeper layer LM sections for both injections of area 3b and area 1. Additional small BDA-labeled endings with diameter < 1 µm under LM (not mapped under LM) were chosen from the series of ultrathin sections cut for the reconstruction of selected large boutons. In the EM series all BDA-labeled small boutons, which could be fully reconstructed in 3D, were used for the analyses. Ten of the 65 BDA-labeled boutons could not be fully reconstructed. Altogether, digital images of the full series of 55 (31 large and 24 small) BDA-labeled terminals were made at 20,000x, 30,000x and 50,000x (EM magnification on the serial ultrathin sections). Only 32 of the 55 fully reconstructed boutons formed an unequivocal synapse characterized by the presence of synaptic vesicles and postsynaptic structures (dendrite or spine and PSD) (Peters and Sethares, 1991). These 32 boutons (16 large including 3 within axonal patches, and 16 small including 2 in axonal patches, based on LM categories) forming clear synaptic contacts were thoroughly analyzed in this study. Many of the other 23 boutons (including 7 localized in axonal patches) contained synaptic vesicles but the PSD could not be unequivocally identified because of a tangential cut or lead staining artifacts (unwanted precipitation). These 23 boutons lacking a clear synaptic contact were excluded from the single and multivariate comparisons, where PSD measurements were included. The 3D reconstruction and measurement of the EM images (3D-EM) were made by Reconstruct software (Fiala, 2005); freely available at (<https://synapseweb.clm.utexas.edu/software-0>). Measurements were made on the reconstructed axonal endings, mitochondria of the boutons and postsynaptic densities. Synaptic vesicles and other small subcellular structures could not be studied because dense NiDAB precipitate obscured the view of many of these structures prohibiting their analyses. The final three-dimensional representation including surface rendering were prepared by Blender software (<https://www.blender.org/>). Raw data is freely available at <https://datadryad.org/stash/dataset/doi:10.5061/dryad.3bk3j9kdv> on Dryad.

Experimental Design and Statistical Analysis—For the ultrastructural analysis, measured variables were: *number* of mitochondria and synaptic contacts, *surface* area of the boutons, PSD and mitochondria and *volume* of the labeled boutons and mitochondria. Additional quantitative variables used for the comparisons were a *shape factor* which in case of the PSD was defined as the aspect ratio of the minimum and maximum diameters of the PSD (Olson, 2011; see also ([https://en.wikipedia.org/wiki/Shape_factor_\(image_analysis_and_microscopy\)](https://en.wikipedia.org/wiki/Shape_factor_(image_analysis_and_microscopy))). For the bouton shape factor we used

sphericity, $\Psi = \frac{(6V)^{\frac{2}{3}}}{A}$, where V is the volume of the bouton and A is the surface of the bouton (Wadell, 1935; <https://en.wikipedia.org/wiki/Sphericity>). A sphericity of 1 indicates a perfect sphere; the smaller the sphericity, the less spherical is the object. Variables such as

unit surface areas and volume were derived as mitochondrion/bouton, PSD/bouton and PSD/mitochondrion ratios. Surface/volume ratios were obtained by using ² surface and ³ volume to maintain dimensional coherence. For univariate comparisons student t-test was used. For multivariate study, we applied principal component analyses (PCA) to clarify the importance of the variables in distinguishing the types of bouton. To overcome the limitation of small sample size of our material and to examine the consistency of the findings, a comparable PCA was performed on a large dataset obtained from human temporal cortex (Yakoubi et al., 2019a,b). The analyses were implemented through MS Excel, Matlab, Dell Statistica™ and GraphPad Prism software.

Results

Areal distribution of the large boutons: light microscopic observations

BDA injections were made into the distal finger pad representation of digit 2 (5 cases) of area 3b or area 1 with one exception receiving BDA injection into the distal finger pad representation of digit 4 of area 1 (one case Mo) (Fig. 1). The injected loci exhibited slowly adapting response to skin indentation. In all cases the core (260 – 403 μm in diameter, 330 μm in average), i.e. the uptake zone of the BDA injection, included the upper layers, while lower layers were also included into the core in cases of area 1 injections (Fig. 1A,B) (for more details see Table 1 in Negyessy et al. (2013) and Ashaber et al. (2014)).

LM was used to reveal whether the distribution of large boutons exhibited distinct intra-areal and interareal projection patterns in the circuitry of areas 3b and 1 of somatosensory cortex. At the light microscopic level the focal BDA injections resulted in labeling a dense meshwork of cortico-cortical axonal fibers radiating from the injection site towards distant cortical regions in area 3b and in area 1 (Négyessy et al, 2013; Ashaber et al, 2014). BDA-labeled axons were decorated by varicosities as well as terminal-like structures, which are typical forms of cortical boutons (Fig. 1C,D). Small varicosities (diameter < 1 μm) (Fig 1C), which formed the bulk of labeled boutons were not mapped, whereas each large anterogradely labeled bouton (diameter > 1 μm) (Fig 1D) was mapped on the serial sections under LM (a total of 237 boutons summed across the three cases). Large boutons with diameter larger than 1 μm appeared both as axonal varicosities and terminal-like endings (Fig. 1D). Large boutons of long range axons (boutons closer than 690 μm to injections sites were not mapped) were found both within the injected area and inter-areally either in area 3b or area 1 (Fig. 1E). It should be noted, that the majority of large boutons (74% of the 237 total population) were found outside axonal patches, which are focal areas (250–350 μm in diameter) of densely ramifying axons with high bouton densities (Négyessy et al, 2013; Ashaber et al, 2014 see also Lund et al. 1993). However, considering that axonal patches form only a small fraction of the total cortical volume analysed, the 26% suggest a relative enrichment of large boutons in the patch regions.

Injection of area 1 resulted in a considerably larger number of the BDA-labeled boutons (a total of 207 boutons summed across the three cases) than the injection of area 3b (altogether 30 boutons in the three cases). This difference was mostly due to greater intra-areal labeling (area 1: 164, area 3b: 17) than inter-areal labeling differences (area 1 to area 3b projections: 43; and area 3b to area 1 projections: 13) (Fig. 1E). The large difference in the absolute

number of the BDA-labeled boutons between the two groups of injections could have been a consequence of the BDA injection of area 1 including lower cortical layers in addition to the upper layers, which was targeted in the case of area 3b injections (Fig. 1B, F). Accordingly, the average number ($\pm$ sd) of boutons on a slide was 3.0 ± 1.2 and 7.9 ± 3.6 following area 3b and area 1 injections, respectively (t-test, $df = 37$, $p = 0.001$).

The relative strength of the inter-areal connections formed by the large boutons was analysed using the formula of Markov et al. (2011) i.e. strength = inter-areal N/(inter-areal N + intra-areal N), where N is the total number of large boutons. A relative strength with a value of 1 would indicate all large boutons were inter-areal; whereas a value of zero would indicate they were all intra-areal. After the area 3b injections just under half of the large boutons were located in area 1 leading to a relative strength of inter-areal connections of 0.46 ± 0.25 (average $\pm$ sd) (Fig. 1F, left). With area 1 injections only about a quarter of the large boutons were located in area 3b leading to a relative strength of inter-areal connections of 0.23 ± 0.08 (average $\pm$ sd) (Fig. 1F, right). These trends observed across cases (t-test, $df = 4$, $p = 0.20$) showed that area 3b injections resulted in a larger fraction of inter-areal labeling than area 1 injections. This was also supported by the significant differences in the total counts of intra-areal and inter-areal large boutons (area 3b injection: 17 intra, 13 inter; area 1 injection: 164 intra, 43 inter; $\chi^2 = 10.807$, $df = 1$, $p = 0.0010$).

Ultrastructural features of BDA-labeled small and large somatosensory cortical terminals in areas 3b and 1

EM was used to determine whether ultrastructural features can distinguish distinct classes of boutons in somatosensory cortex. In addition to large boutons, small BDA-labeled endings, which were smaller than $1 \mu\text{m}$ and not mapped under LM, were also serially reconstructed by EM. Altogether 55 BDA-labeled boutons (31 large and 24 small), which provided a representative sample of intra-areal and inter-areal boutons observed after area 3b and area 1 injections, were used to analyse basic ultrastructural properties (Fig. 1E, Table 1).

At the EM level BDA-labeled small terminals contained round synaptic vesicles and formed Gray's type I (asymmetric) axospinous synaptic contacts (Fig. 2A–C). Large endings as determined by their LM size, also established asymmetric synaptic contacts with dendritic spines or shafts (Fig. 2D–F). Both small and large endings formed simple and complex perforated asymmetric synapses (Fig. 2). Interestingly, under EM, 5 of the large varicosities were exceptionally large, appeared non-synapsing and lacking synaptic vesicles. These 5 “giant” axonal swellings, observed in both areas 3b and 1, were excluded from further analyses reducing the number of large endings to 26 (Table 3).

For quantitation of the ultrastructural aspects of the boutons, a 3D reconstruction was performed as shown in Figure 3. As boutons can enclose invaginations of non-labeled structures without forming synaptic contact (Fig. 2C and 3D) and exhibit irregular shape (Figs. 2 and 3) only full 3D reconstructions allow comparisons of their size and shape. Also, in addition to single synapse (Figs. 2 and 3G–J), boutons also formed multiple synapses with more than one postsynaptic structures (Fig. 3A–D). Consequently, a number of features of the boutons were measured (see Methods).

Correlation of surface and volume of BDA-labeled boutons

Surface and volume measurements were used to evaluate the validity of the LM and EM categorizations to divide the boutons into two groups, small and large. In addition, the unit surface area (surface/volume) of the boutons, which can indicate the relative weight of membrane bound (e.g. receptors and ion channels) or cytoplasmic second messenger cascade (e.g. via Ca^{2+}) presynaptic functions, was also compared between the groups. Figure 4 shows bouton volume (A), surface (B) and surface/volume ratio (C) measured and calculated after 3D-EM reconstruction of the 50 boutons (26 large and 24 small, based on LM). Comparison of surface and volume of the large boutons revealed the presence of an outlier (Fig. 4A,B). Notably, the surface/volume ratio of this extra-large bouton did not differ from the majority of the large population (Fig. 4C). Surface (average $\mu\text{m}^2 \pm \text{sd}$ for small: 1.0 ± 0.5 , large: 4.5 ± 2.7) and volume (average $\mu\text{m}^3 \pm \text{sd}$ for small: 0.1 ± 0.07 , large: 0.7 ± 0.6) differed significantly between the small and large boutons both before (t-tests, $\text{df} = 48$, all P s < 0.02) and after omitting the outlier (t-tests, $\text{df} = 47$, all $p < 1.5 \times 10^{-5}$) (Bonferroni correction for multiple comparisons) (Fig. 4A–B). Surface/volume ratios computed by using transformed data (² surface and ³ volume) (average $\pm \text{sd}$ for small: 2.2 ± 0.2 , large: 2.5 ± 0.4) also differed significantly at $p = 0.017$ obtained by Bonferroni correction (t-test, $\text{df} = 48$, $p = 0.016$) (Fig. 4C). These findings provided support for the classification of boutons on the basis of size at the LM level.

The relationship between the surface and volume of the boutons were further investigated by correlation analysis (Fig. 4D–F, Table 2). The analysis showed that volume and surface area of the boutons are highly correlated (Spearman $R = 0.95$) and with the exception of a single case, grouped the boutons into two clusters similar to the LM categorization (Fig. 4D). Note again, that the 5 non-synapsing „giant” varicosities were not included into the analyses (Table 3). The single exception was the outlier exhibiting the largest volume and surface areas (Fig. 4A,B,D). In Figure 4E, enlarging the scales and excluding the outlier further revealed the clustering of small and large types of terminals. As shown on Figures 4G and H, no boutons were found with surface area between $2.4\text{--}2.9 \mu\text{m}^2$ and volume between $0.5\text{--}0.6 \mu\text{m}^3$. Notably, surface area was found as a better measurement for classifying the boutons because volume measures exhibited larger scattering making it difficult to determine the size distinguishing the small and large groups (Figs. 4E,G,H).

The surface and volume of the mitochondria exhibited similar high correlation to that of the boutons (Table 2). Also, mitochondrial surface highly correlated to the surface of the boutons. Although, the volume of boutons and mitochondria did not correlate as well to each other, this correlation was also significant (Table 2). However, PSD area correlated neither to the surface of boutons nor to that of mitochondria (Table 2). This raised the question about the role of PSD in classifying the boutons.

The LM and EM classifications were not in full agreement as 12 of the large terminals grouped according to LM criteria were better classified as small boutons based on the correlation of surface area and volume of the bouton (Fig. 4D,E, Table 3). Note, that the surface and volume of the large (by LM criteria) regrouped boutons were among the largest of the small group (by EM grouping, see open and filled circle symbols representing boutons with surface area around $2 \mu\text{m}^2$ on Fig. 4E). Furthermore, boutons with or without

mitochondrion or the presence or absence of PSDs distributed similarly across groups indicating that these factors do not play a role in the grouping of boutons (Fig. 4E,F). These observations indicate that in contrast to the one-dimensional measure by light microscopy (diameter), the correlation of volume and surface and thus the use of 3D electron microscopy provides a better tool for the classification of cortical boutons by size.

Basic synaptic properties of PSD forming boutons classified by 3D-EM

Up until this point our dataset included all boutons (withouth the non-synapsing 5 “giants”) where the size could be reconstructed to their full extent. However, the PSD could not be completely delineated for the complete population due to tangential cut or lead staining artifacts (unwanted precipitation). Consequently, 18 boutons were excluded from further analysis, where morphological features related to the dynamic properties of the synapse, such as plasticity and efficacy of synaptic transmission, were of major interest. Notably, the discarded boutons contained synaptic vesicles supporting the validity of the above analyses. As shown in Table 3, out of the 50 reconstructed boutons, 32 formed synaptic contacts via clearly identifiable PSD. As the single PSD forming giant bouton was considered as an outlier and excluded, 31 axon terminals were thoroughly analyzed for their quantitative 3D electron microscopic features (15 large and 16 small).

The 3D-EM classification and the omission of boutons without clearly demarcated PSD changed the number of boutons in the small and large groups (Table 3). Considering only axon terminals with PSD half (8/15) of the boutons identified as large by LM were reassigned into the small group following the 3D-EM analysis (Table 3; see also Fig. 4D,E). All of the redistributed boutons were inter-areal endings following area 3b injections (Table 4). After redistribution, the majority of large boutons were found inter-areally and only a single bouton was labeled intra-areally. Altogether in the following single and multivariate analyses of the 3D ultrastructural features we studied 31 boutons, 7 large and 24 small boutons including the 8 reclassified; 15 were intra-areal (7 from area 1 and 8 from area 3b injections) and 16 were inter-areal (9 from area 1, and 7 from area 3b injections) axon terminals (see Large and Small 3D-EM groups in Table 4).

Figure 5 shows the basic synaptic characteristics of the 24 small and 7 large boutons with unequivocally delineated PSD. Multiple PSDs were formed at low frequency in both groups (Fig. 5A). In contrast, perforated PSDs were observed with a relatively high incidence in approximately 60% and 30% of the large and small boutons, respectively (Fig. 5B). Large boutons formed complex perforated and multiple PSDs at a higher ratio than small boutons (Fig. 5A,B). In regard to mitochondrial content, an equally large proportion, roughly 70% of the small and large boutons contained mitochondrion (Fig. 5C). The giant varicosity, which established synaptic contact, formed multiple and complex, perforated PSDs and contained multiple mitochondria (not shown). These findings suggest that the type of PSD vary with the size of boutons.

Single and multivariate comparisons of 3D ultrastructural features of the boutons

Single variable pairwise comparisons—Ultrastructural features of the large and small boutons were compared to see which elements serve as distinguishing components. As

shown in Figure 6, the size of boutons and mitochondria exhibited significant differences between the two groups, whereas the size of PSDs as well as the shape of boutons and PSDs did not. The highly significant differences of the surface and volume supported the correlation based grouping (Fig. 4E) of the small vs. large endings (t-test, $df = 29$, $p = 10^{-7}$ for both comparisons, Fig. 6A,B). Also, similar to the LM grouping (Fig. 4C), the ratio of the bouton surface area to bouton volume were different between the large and small groups (t-test, $df = 29$, $p = 0.018$; Fig. 6C), which support that a disproportional enlargement of the surface area plays a role in the specialization of cortical boutons. The size of mitochondria including both the surface (t-test, $df = 21$, $p = 0.0001$) and volume (t-test, $df = 21$, $p = 0.00008$) were significantly larger in the large than the small endings (Fig. 6D,E). In contrast, the size of the mitochondria relative to that of the bouton did not differ significantly, even though the ratios tended to be larger for the small than large boutons (t-test for the surface: $df = 21$, $p = 0.53$, for the volume ratios: $df = 21$, $p = 0.2$; Fig. 6F,G). These results indicate that in addition to the surface and volume of the boutons, mitochondrial surface and volume are also major distinguishing variables of the small and large boutons.

The PSD area formed by the small and large boutons was similar (t-test, $df = 29$, $p = 0.87$) (Fig. 6H). However, the area of PSD expressed relative to the unit surface area of the bouton was significantly larger for the small than the large bouton group (t-test, $df = 29$, $p = 0.008$; Fig. 6I). In contrast, the size of PSD relative to the surface area of the mitochondrion only tended to be different (t-test, $df = 21$, $p = 0.052$; Fig. 6J). These findings point to the importance of relative rather than absolute size of the PSD in synaptic transmission.

The shape factors (see Methods), which quantified how similar PSDs were to circle and boutons to a sphere, found that PSDs in both groups of boutons exhibit similar circular shapes (t-test, $df = 29$, $p = 0.41$; Fig. 6L). Similarly, bouton shape did not differ significantly (t-test, $df = 29$, $p = 0.09$; Fig. 6K). However, large boutons tended to be less spherical than small endings probably due to the invaginations and protrusions. Overall, it is worth noting that mitochondria and PSDs, when scaled to the size of the bouton, tended to encompass a larger fraction of the small boutons than the large boutons (Fig. 6F,G,I,J) suggesting that the differences in bouton surface and volume play a major role in the specialization of synaptic transmission.

It should be noted that, except the surface per volume ratio of the boutons (Fig. 6C) and PSD per bouton surface area ratio (Fig. 6I), Bonferroni correction of the original 0.05 significance level, which yielded a p value of 0.004, did not change the significance of the comparisons. Larger sample size is needed to clarify the differences of these values in the two groups.

Multivariate analyses—PCA was used to explore the relative and combined significance of the variables in the classification and consequently the physiological properties of the BDA-labeled small and large boutons. PCA was performed on a full set of variables (12 in total) including both measured (surface and volume of the boutons, mitochondria and PSDs) and those derived as the ratios of surface and volume data as well the shape factors. PCA determines the importance of variables by reducing the effect of redundancy in any correlations in the data. To normalize the different measures for the PC analysis the

numerical values of the ultrastructural features were transformed such that each variable had a mean of zero and unit standard deviation. The analysis yielded two major principal components, which together explained 55% of the variance in the data (Fig. 7A). The variables accounting for most of the variability were the surface of the mitochondria relative to the corresponding measure of the boutons and the total surface area and volume of the mitochondria within the boutons (Fig. 7B). The least important variable was the shape factor of the PSD. Notably, the surface per unit volume of the bouton also exhibited low importance as a distinguishing variable. Figure 7C shows for the first 2 principal components that variables related to mitochondria vary independently from the other variables further suggesting that mitochondrial measures are important in classifying cortical boutons. It is notable that the indices about the size of the boutons including both the measured (surface and volume) and the derived (surface/volume ratio) variables vary independently from the rest of the variables. The PCA indicated that our sample of boutons form a relatively homogenous group without outliers (Fig. 7D). Interestingly, there is a clear tendency of forming two clusters, as large boutons (open circles) tended to group separately from the small boutons (black rectangles). The PCA analysis showing a tendency to group the small and large boutons separately using only the first two principal components attest to the power of bouton and mitochondrion size variables in distinguishing large and small boutons.

Considering the small sample size especially in the large group of boutons we checked the generality of our results by extending the analysis to datasets obtained in the human temporal cortex (Yakoubi et al., 2019a,b). Lübke and colleagues (Yakoubi et al., 2019b) showed that boutons are larger in layer 5 (L5) than in layer 4 (L4). Therefore, we were interested to see if boutons are clustered in regard to the size (defined by the surface area and volume of boutons) when several other variables are included in the analysis. The dataset consisted of 296 synaptic boutons, 150 from L4 and 146 from L5. The set of ultrastructural measures obtained from the temporal cortex was similar to that used in the above analysis in somatosensory cortex with one exception, the human data does not include mitochondrial surface values. Without the surface of mitochondrion, the human data consisted of 9 variables (vs. 12 variables, compare Fig. 7B and Fig. 8B). Note also, that in the human dataset circularity and sphericity correspond to the shape factors of PSDs and boutons used for the non-human primate data. Similar to the non-human somatosensory cortex, the first two principal components explained the majority of variance (63%) in the data from the human temporal cortex (Fig. 8A). The major distinguishing variables most notably included bouton surface area, and the size of mitochondria in terms of volume followed the bouton related measures (Fig. 8B). Similar to the monkey somatosensory cortex, less variability could be accounted for with PSD related measures in the human temporal cortex. The volume of mitochondria relative to that of the bouton was the least important variable (Fig. 8B). Considering the two largest principal components, the loading factors (Fig. 8C) show that size measurements including that of the boutons, mitochondria and PSDs vary together and are mostly responsible for the differences of synaptic boutons in the dataset. These size measures, except mitochondrion/bouton volume varied relatively independently from the rest of the variables including the shapes of boutons and PSDs as well as derived variables of PSD/bouton and surface/volume of boutons. Most interestingly boutons of the two layers

tended to form 3 clusters, one consisted by L4 boutons, another including L5 boutons and the 3rd one containing boutons from both layers (Fig. 8D). This observation is in agreement with the finding of Yakoubi et al. (2019b), who reported that the size of L5 boutons are larger than that of L4, and extends it by showing that cortical boutons form morphologically heterogeneous groups. To see if size plays a major role in the segregation of boutons, surface versus volume was plotted, which resulted in a trend toward a triple segregation of boutons similar to that shown on the case-wise PCA analysis (Figs. 8D, 9). Also, similar to the non-human data surface and volume of boutons were strongly correlated in human temporal cortex (Spearman $R = 0.9$).

To make the analysis of the two datasets comparable, we repeated the PCA on the non-human somatosensory cortical data after omitting variables based on mitochondrial surface measurements. With the reduced set of 9 variables the first two principal components explained a similar amount of variance (61%) to that found in the human dataset. Excluding variables changed the significance of the remaining variables in making distinctions between different boutons (Fig. 10A). Notably, variables appeared to have very similar importance as found in the human data: bouton related measures exhibited the most, and mitochondrion/boutons volume the least power (compare Fig. 8B and Fig. 10A). Importantly, using only 9 variables, small and large boutons tended to cluster similarly as found by using all 12 variables (Fig. 10B). This result strongly support that size related measures of boutons (surface and volume) play determining role in defining bouton types.

Discussion

Overall, in somatosensory cortex the 3D-EM revealed 2 major classes of boutons based on the groupings observed in the correlation of bouton volume and surface area and on the ultrastructural complexity of the axonal ending. PCA supported the results of correlation analyses and added the size of mitochondrion (both absolute and relative to the bouton surface) as important variable in classifying cortical boutons. This observation was in full correspondence to single variable comparisons showing that size of the bouton and mitochondria are major determining factors of synaptic specialization. On the other hand, PSD related variables including the size per unit surface area of the bouton exhibited relatively small distinguishing power. However, it should be noted that in the PCA analyses including boutons with and without mitochondrion, i.e. the mere presence of this organelle could have increased the importance of mitochondrial related variables at the expense of others, most notably PSD related measures as distinguishing factors of bouton types. Considering measures of bouton size, in all PCA analyses it exhibited high power suggesting that as a classifying factor bouton size is robust to changes of the other variables. Accordingly, multivariate analyses of 3D ultrastructural variables of boutons of human temporal cortex also indicated that the size of boutons is a major classifying factor of boutons in the cerebral cortex. These findings support that in the cerebral cortex synaptic interactions are mediated via synapses formed by Class 1 and Class 2 boutons (Petrof and Sherman, 2013). Also, the observation of differences in the scaling of mitochondria and PSDs with bouton size suggest a complex role for mitochondria in synaptic transmission.

Methodological considerations

Our BDA labeling cannot completely exclude the possibility of backfilling thalamocortical endings exhibiting large size, forming multiple PSDs and having large mitochondrial content (Negyessy and Goldman-Rakic, 2005). Also, regular sampling of the LM series prevented tracing the axons forming large boutons back to the origin due to cuts of segments running out of the section plane. However, we have described thick axons both projecting intrinsically and forming reciprocal inter-areal pathway in somatosensory cortex (Pálfi et al., 2018). As thick axons form large boutons (Anderson and Martin, 2006; Innocenti and Caminiti, 2017) these findings suggest that at least a considerably portion of the large boutons studied here have a cortical origin. Also, we observed a very low proportion of boutons forming multiple PSDs. It is known that area 3b has a stronger thalamic input than area 1 (Shanks and Powell, 1981; Iwamura et al., 1983; Jones, 1983; Cusick et al., 1985), which, in case of trans-thalamic labeling of the reciprocal thalamocortical endings could result in a high ratio of intra-areal large boutons in area 3b and a low ratio of large intrinsic terminals in area 1, just the opposite found in this study. These observations make unlikely that our findings are largely a result of labeling of thalamocortical endings. Furthermore, the different areal and laminar distribution of the cortical projection neurons (Pálfi et al., 2018) and large boutons suggest that backfilling of the boutons could not have been a significant factor.

The small sample of large boutons was another major limitation of this study. However, the agreement of the results with the correlation analysis, which included a much larger sample, suggests that PCA is a powerful technique especially in case of a relatively small sample size. Similarly, the results of PCA and single variable comparisons made on the same samples were in good agreement further supporting the usefulness of PCA. Furthermore, our observations in primate somatosensory cortex regarding the dominant role of the size of bouton as a distinguishing feature was corroborated by the large human sample from temporal cortex.

Classification of the cortical axon terminals

With both a correlation analysis and PCA, we found distinct groups of small and large endings with features similar to Class I and Class II boutons (Petrof and Sherman, 2013). There was a high correlative relationship between bouton surface area and volume which can be used to group boutons, providing an objective tool for classification of cortical axon terminals. The sensitivity of the correlation analysis was supported by the regrouping of boutons identified as large by LM into the small category. The discrepancy between the EM and LM classification was probably due to the complex shape of the terminals, which can appear larger (or smaller) in their 2D projection under LM as compared to EM especially near the resolution limit of LM. The reliability of the correlation analyses was confirmed by the PCA analysis, which resulted in a similar grouping of the small and large types of boutons. The high correlation of bouton surface and volume also indicate their joint scaling, which suggest a tight coupling of membrane bound, and cytoplasmic second messenger processes in boutons. While the single variable comparison showed some trend for a higher surface to volume ratio in the group of small boutons, the difference did not reach the level

of significance. It has to be added, that a definitive answer to this question can only be given by using a sample size larger than used in this study.

Further evidence in favor of the importance of bouton size in classification of cortical axon terminals was obtained by studying human temporal cortex (Yakoubi et al., 2019a,b). One may argue that the PCA and correlation between surface and volume did not result in a clear segregation of the small and large boutons, which became evident only when the laminar location of the boutons were indicated in the human sample. However, it is important to note that in contrast to the monkey somatosensory cortex data set, where the origin of boutons was clearly defined by way of tract tracing, the human data included boutons of diverse, undetermined afferent pathways. In light of this, the tendency of segregation of boutons of the human temporal cortex is even more compelling and suggests that identification based on the size of boutons is a valid and universal approach. These findings together point to the importance of quantitative 3D electron microscopy in typifying boutons in the cerebral cortex and also to the usefulness of bouton size as a distinguishing variable based mostly on its dependence on several other structural features such that the size and number of mitochondria, synaptic vesicles and PSD.

Surface and volume measurements identified two classes of boutons (ignoring the giant, non-synaptic varicosities). The small type, which is the most abundant, forms single, mostly axospinous synapse where most contain mitochondrion. This type is reminiscent of the Class 2 axon terminal according to the definition of Sherman and colleagues (Covic and Sherman, 2011; Petrof and Sherman 2013). The second group consisted of larger boutons, which, in contrast to the small type, is associated with perforated PSDs and also contained mitochondria. The ultrastructural features of this type of bouton is similar to the Class 1 of Sherman et al. (Petrof and Sherman, 2013). The characterization of the Class 1 bouton is defined primarily on the basis of thalamocortical projections and large, layer 5 corticothalamic axon terminals. In the cortex of non-human primates thalamic endings form multiple synapses and contain several mitochondria similar to synapses of feedforward cortical afferents and amygdalo-hippocampal pathways (Anderson et al., 1998; Negyessy and Goldman-Rakic, 2005; Anderson and Martin, 2006; Zikopoulos and Barbas, 2007; Wang and Barbas, 2018). In contrast, in somatosensory cortex only a small proportion of boutons form multiple PSDs. Interestingly, the PSD formed by thalamocortical and feedforward cortical terminals exhibit in many cases complex, perforated shapes (macaque: Anderson et al. 1998; Négyessy and Goldman-Rakic, 2005; Zikopoulos and Barbas, 2007; mouse: Rodriguez-Moreno et al. 2017) as found in the case of the large boutons here. These observations suggest that large endings in somatosensory cortex share many features with the Class 1 cortical and thalamocortical synapses. These similarities and previous findings suggest that synapses of large boutons in somatosensory cortex have high efficacy and evoke large postsynaptic responses (Amiati, 2001; Chung et al., 2002; Ganeshina et al. 2004; Groh et al., 2008; Pelzer et al, 2017).

PCA indicated that the size of mitochondria was also a distinguishing factor in classifying the cortical axon terminals. In agreement with the results of PCA analyses single variable comparisons also showed that surface and volume of mitochondria exhibit noticeable variation among the axonal endings and revealed that the size of mitochondria scales with

the size of boutons, as shown previously (Pierce and Lewin, 1994; Germuska et al. 2006; Eyre et al., 2007; Rodriguez-Moreno et al., 2018). Since mitochondria serve both as source of energy as well as Ca^{2+} reservoir it potentially determines the dynamics of synaptic transmission (Vos et al., 2010). Although, it is unknown whether in large endings the increased size of mitochondria signifies high need of energy or Ca^{2+} or both, these findings add further evidence that activity can shape the size of boutons (Pierce and Lewin, 1994). In small boutons the trend for a larger relative mitochondrial size per unit size of bouton raises the possibility of a somewhat larger need for mitochondrial functions than in the large boutons. A similar tendency is shown in the size of PSD relative to the unit mitochondrial size. However, compared to the large boutons, the smaller size of mitochondria in the small boutons suggests that mitochondria are not specifically involved in synaptic functions such as Ca^{2+} dependent signaling. In this context, the significantly larger PSD per unit surface area of the small boutons suggests a higher synaptic activity, hence the increased need of energy in the small boutons than in the large ones.

An additional noticeable finding of the present study was that the size and probably also the shape of the PSD are invariant properties. Size invariance of the PSD was also found previously (Germuska et al., 2006; Karbowski, 2014; Rodriguez-Moreno et al. 2018) (but see Eyre et al., 2007; Hsu et al., 2017). Consequently, the relative size of the PSD (PSD area/bouton surface ratio) is larger for the small boutons than for the large ones supporting the assumption that the larger relative mitochondrial size is the result of the higher need of energy in the small boutons.

Synaptic organization of the cortico-cortical pathways of somatosensory cortex

That connections of somatosensory cortex are formed by small and large axon terminals is in agreement with previous observations in other primate cortical areas (Anderson and Martin, 2009; Covic and Sherman, 2011; Innocenti and Caminiti, 2017). However, in contrast to the reciprocally connected V1 and V2 of the macaque monkey, where only the V2 to V1 projection is formed by small and large boutons (Anderson and Martin, 2009) our findings indicate that the reciprocal inter-areal connections are formed by boutons of heterogeneous size in both directions. Also, this is the first study showing the parallel existence of intrinsically projecting, long range synaptic pathways formed by small and large boutons within a cortical area. Moreover, our LM observations found that large inter-areal endings form a smaller population than that of the intra-areal large boutons. These observations on intrinsic and inter-areal pathways of somatosensory cortex of the squirrel monkey indicates species and/or regional differences (Hsu et al., 2017) in the organization of large boutons.

In cortical hierarchy area 1 is positioned above area 3b (Sur et al., 1980, Iwamura, 1998; Kaas, 2004). The intra-areal and reciprocal inter-areal distribution of large boutons as shown here adds further support to the increasing body of evidence that synaptic organization does not simply reflect the hierarchical organization of cortical pathways (Anderson and Martin 2009; Covic and Sherman, 2011). The somatosensory cortex is functionally divided into slowly and rapidly adapting as well as Pacinian modules (Friedman et al., 2004). Short term synaptic depression characterizing driver-like functions would be the most compatible with the rapidly adapting and/or Pacinian pathway in the somatosensory cortex, which provide

transient responses to peripheral stimuli (Amitai, 2001; Chung et al., 2002; Groh et al., 2008; Covic and Sherman, 2011; Petrof et al., 2015; Pelzer et al., 2017). However, such rigid functional segregation of cortical boutons to small, modulator-like synapses in slowly adapting pathway and large, driver-like synapses in the rapidly adapting and Pacinian pathways is unlikely. Thalamocortical afferents are of the driver type exhibiting short term depression (Chung et al., 2002; Petrof and Sherman, 2013) and target both magno, and parvocellular pathways in visual cortex. Also, our injections were made in the slowly adapting modules of the somatosensory cortex. It is more plausible to assume, that large, driver-like terminals transmit information to hot-spots of the somatosensory cortical representations while the more numerous small, modulator-like terminals transmit information to the surround of receptive fields (Favorov et al., 1987; Reed et al., 2010). However, small and large boutons originating from the same injection site were localized in a close vicinity of each other, i.e. in the same ultrathin sections, which questions the idea of functional specialization of large and small boutons according to a simple center-surround organization of receptive fields. Further studies are needed to determine the role of these two classes of boutons in the processing of somatosensory information.

In a broader perspective our findings that Class 1-like synapses are formed by long-range intrinsic projections as well as by afferents across the cortical areas irrespective of the area of origin is more compatible with the log-dynamic brain theory (Buzsáki and Mizuseki, 2014). Accordingly, the large, Class 1-like boutons formed a minor fraction of the BDA-labeled boutons, where small Class 2-like endings were far more numerous. Considering the existence of quick somatosensory cortical pathways formed by thick axons (Palfi et al., 2018), which are known to form large boutons (Innocenti and Caminiti, 2017), our findings suggest that large boutons represent synapses of the “fast firing minority” of excitatory neurons (Buzsáki and Mizuseki, 2014). Thereby, large boutons are responsible for a quick and faithful dissemination of tactile information across the full circuitry of somatosensory cortex, which represent a “best match” (Buzsáki and Mizuseki, 2014) of the input. Then, as predicted by the log-dynamic brain theory, the functional representation is refined by the slow firing excitatory neurons communicating via small boutons. The refinement is perhaps the result of modulating the gain and not the selectivity of circuit activity driven by large boutons (Miller, 2016). These could lead to the emergence of the different functional properties of area 3b and area 1 neurons.

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Abbreviations:

ABC	avidin biotin complex
AP	anteroposterior

BDA	biotinylated dextran amine
3D	three dimensional
3D-EM	three-dimensional reconstruction by electron microscopy
CAR	Contour Alignment Reconstruction
DAB	diaminobenzidin
ECG	Electrocardiogram
EM	electronmicroscopic
ET-CO₂	amount of carbon dioxide (CO ₂) exhaled
IACUC	Institutional Animal Care and Use Committee
IOS	intrinsic signal optical imaging
LM	light microscopic
ML	mediolateral
NIH	National Institutes of Health
PB	phosphate buffer
PSD	postsynaptic density
PCA	principal component analysis

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Figure 1.

Anterograde BDA labeling and distribution of large boutons at the light microscopy level. **A**, **B**: Injection sites. Representative light microscopic images of the injection sites (**A**) taken from the sections labeled by stars in the graphical reconstructions (**B**) in the 6 cases. The lateral spread of the core and halo regions are shown by black and grey, respectively, across the sections where BDA injections could be identified. White dots indicate the electrode track. Upper panels: area 3b injections, lower panels show area 1 injections. Depth of the uppermost and lowermost sections from the pial surface, with clearly detectable BDA

injection site are also indicated. Note the injection of the full cortical thickness in cases P and Mo with area 1 injections. **C, D:** Small and large boutons identified by light microscopy. Representative light microscopic image showing numerous thin fibers decorated by small boutons (diameter $\approx 1 \mu\text{m}$) in a terminal axon arborization patch of area 3b (C). Arrowheads show terminal-like structures with stalk, and arrow indicates varicosity. An example of an axonal branch bearing 2 small varicosities (arrows) are shown in the inset. Example of large boutons (diameter $> 1 \mu\text{m}$) appearing as varicosities of long-range intra-areal and inter-areal horizontal axonal fibers in area 1 (D). **E:** Distribution of the large boutons (diameter $> 1 \mu\text{m}$, each marker corresponds to one large bouton) after aligning the merged series of 6 cases using the injection sites (i.j.) and the border between area 3b (a3b) and area 1 (a1) as fiducial landmarks. Only boutons in areas 3b and 1 are shown. Grey squares show large boutons labeled by area 3b injections and black dots show boutons labeled after area 1 injections. Large markers indicate boutons reconstructed by EM. Injections were made into digit 2 (d2) distal finger pad representation except one case with area 1 injection, where digit 4 (d4) distal finger pad representation was injected. Note that the distance of bouton closest to the injection site was $690 \mu\text{m}$. Dotted lines: areal borders, arrows: injection sites, sc: central sulcus. r: rostral, m: medial. Scale bars: $200 \mu\text{m}$ on A and B, $5 \mu\text{m}$ on C and D and 1 mm on E. **F:** Areal distribution of large boutons in areas 3b and 1. Inter-areal ratio represents the inter-areal fraction of the total number observed in the injected and target areas. Squares indicate individual cases and stars show averages.

Figure 2.

Ultrastructural features of BDA-labeled somatosensory cortical endings. **A-C:** Electron microscopic cross sections of small boutons (diameter $\approx 1 \mu\text{m}$ light microscopy size) forming synapses with dendritic spines (sp). **D-F:** Electron microscopic cross sections of large boutons (diameter $> 1 \mu\text{m}$ light microscopy size) forming axospinous (E, F) and axodendritic (D, d: dendrite) synapses. All BDA-labeled boutons establish asymmetric synaptic contact (arrowheads) in the forms of simple (A-C, E) and complex perforated synapses (D, F). Note the lack of mitochondrion (m) in some boutons (A). Note also that boutons can contain more than one mitochondrion (D, F). In A, and also on D and F, note the unstained synaptic vesicles (v) appearing as white dots in the dark NiDAB precipitate, and aggregating near the synaptic contact in the bouton. On C note an invagination (arrow), which lacks synaptic membrane specialization within the bouton. Scale bar: $0.5 \mu\text{m}$ on A- D, F and $1 \mu\text{m}$ on E.

Figure 3.

Two examples of serially reconstructed labeled large boutons. **A:** Light microscopic image of a large axonal varicosity examined ultrastructurally in B-D. **B:** Boundaries of the reconstructed bouton based on the serial ultrathin sections are shown. Arrows labeled by D₁, D₂ and D₃ identify sections shown in D. **C:** Three-dimensional structure of the synaptic organization of the labeled bouton (beige) is shown after surface rendering with opaque (C₁) and transparent coloring (C₂). Spines are shown in grey, mitochondrion is blue and synaptic membrane specialization is green. **D:** A short sample of the electron microscopic series

showing two asymmetric synapses (arrowheads) formed by the labeled bouton with dendritic spines (sp1, sp2). Note numerous synaptic vesicles (white dot-like structures) and an invagination (arrow see also on B), which lacks synaptic membrane specialization within the bouton. The bouton also contains a mitochondrion (m). **E-G**: Another example of the serially reconstructed labeled large bouton. Conventions are the same as B-D. Note asymmetric synaptic membrane specialization on F (arrowheads). **H**: For comparison a 3D structure of a small bouton is shown with an opaque (H_1) and a transparent beige coating surface (H_2). The bouton contains a mitochondrion (blue) and creates a synapse (green) with a spine (grey). Scale bars: 5 μm on A and 1 μm on B-H.

Figure 4.

Comparing the surface and volume of the BDA-labeled bouton groups. **A:** Surface, **B:** Volume and **C:** Surface/volume ratio of small, and large boutons. To calculate ratios the data was transformed (2 surface and 3 volume) to maintain dimensional coherence. Plots show individual boutons (open circles) and average (horizontal bar). The small solid symbol in the center of the open markers label boutons with well circumscribed PSD. Note an outlier (filled circle) exhibiting very large surface (A) and volume (B). However, this bouton (large transparent circle) exhibited similar surface/volume ratio to the rest of the population (C). **D:** Correlation of volume and surface resulted in the grouping of small (open triangles) and large (open circles) boutons. The outlier with very large surface (A) and volume (B), is

shown by the black circle. Solid symbol in the center of a marker indicate boutons with PSD. **E:** Note that boutons with and without PSD exhibit similar distributions and high correlation of the surface and volume (PSD: $R = 0.90$, noPSD: $R = 0.98$). Note the regrouping of some LM large boutons into the cluster of small boutons. **F:** Distribution of small boutons lacking (grey triangles) or containing (black triangles) mitochondrion also exhibit high correlation of the surface and volume ($R = 0.77$). **G,H:** Histograms showing the surface and volume distribution in the population of small and large boutons. For better visibility giant varicosities were not included. The absence of data at $2.4\text{--}2.9\text{ }\mu\text{m}^2$ and $0.52\text{--}0.62\text{ }\mu\text{m}^3$ revealed the separation of clusters.

Figure 5.

Frequency of basic ultrastructural features of the BDA-labeled boutons grouped on the basis of surface-volume correlation. **A:** Ratio of boutons forming multiple synaptic contacts via multiple PSDs. **B:** Ratio of synaptic contacts with perforated PSDs. **C:** Ratio of mitochondrion containing boutons. The single giant bouton, which established synaptic contact was not included in the comparisons.

Figure 6.

Comparison of ultrastructural features of the small and large boutons. **A:** Bouton surface, **B:** Bouton volume, **C:** Bouton surface per volume. To calculate the ratios data was transformed (2 surface and 3 volume) to maintain dimensional coherence. **D:** Mitochondrial surface per bouton, **E:** Mitochondrial volume per bouton, **F:** Mitochondrial surface per bouton surface, **G:** Mitochondrial volume per bouton volume, **H:** PSD surface, **I:** PSD surface per bouton surface, **J:** PSD surface per mitochondrial surface, **K:** Bouton shape factor, **L:** PSD shape factor. Note, that the shape factor of PSDs did not differ significantly from 1 (one sample t-

test, $df = 29$, $p = 0.4$ with a mean $\pm$ sd of 1.1 ± 0.7), while the shape factor of boutons was significantly smaller than 1 (one sample t-test, $df = 29$, $p = 0.04$ with a mean $\pm$ sd of 0.9 ± 0.2). Mean is shown by horizontal bar and circles represent individual boutons. Asterisk indicates significant difference for the non-corrected p values. **: $0.005 < p \leq 0.01$. ***: $p \leq 0.005$.

Figure 7.

Results of the PCA analyses. **A:** Scree plot showing the eigenvalues and the percentage of variance explained by the 11 principal components. **B:** The importance of the variables measured by the modeling power, which is defined as the explained standard deviation. **C:** Distribution of the loading factors (transformed values showing the contribution of the variable to the PCA model) P1 and P2 for the first and second principal components. The greater a variable is away from the origin, the more influential that variable has. Diagonal positioning in opposite quadrants means negative correlation between the variables. **D:** Case-wise analysis shows the tendency of grouping the black squares and open circles, which represent small and large boutons, respectively, along the two principal components. Distribution of the scores (distances of transformed values of the variables from the origin along the PCs) of boutons for the first principal component (T1) plotted against the scores for the second principal component (T2). Ellipse outlines $\pm 3sd$ and indicates that there was no outlier in the dataset. M/B sf: mitochondrial surface/bouton surface, M/B vol: mitochondrial volume/bouton volume, B shf: bouton shape factor, B sf/vol: bouton surface/volume, PSD/M sf: PSD surface/mitochondrial surface, PSD/B sf: PSD surface/bouton surface, PSD shf: PSD shape factor.

Figure 8.

Results of the PCA analyses of boutons from layers 4 and 5 of the human temporal cortex. **A:** Scree plot, **B:** Variables importance, **C:** Distribution of the loading factors and **D:** Case-wise analysis. Boutons of layer 4 are marked by black rectangles and boutons of layer 5 are shown by open circles. Note the appearance of outliers, which all except one belong to boutons of layer 5. sph: sphericity, circ: circularity. All other conventions are the same as in Figure 7. Note that this analysis was based on 9 variables as opposed to the 12 variables used for analyzing the BDA labeled boutons. Note also on D the outliers, which is consisted of layer 5 boutons (circle with cross) with one exception from layer 4 (cross with the oblique rectangle in the center).

Figure 9. Correlation of volume and surface of boutons of layers 4 and 5 of the human temporal cortex. L4: layer 4 (rectangles), L5: layer 5 (circles). Note that the highest and lowest values are composed almost exclusively of L5 and L4 boutons, respectively.

Figure 10.

Results of the PCA analyses of BDA labeled boutons using a set of variables matching those used for analyzing the human data. **A:** Variables importance, **B:** Case-wise analysis. Black rectangles: small boutons, open circles: large boutons. Conventions are the same as in Figure 7.

Table 1.

Light microscopic areal distribution of the number of boutons used for 3D-EM reconstruction. Note that small boutons were not mapped by LM. The localization of small boutons were determined on the basis of the localization of the EM sections containing the large boutons. Intra-areal: within the area of injection, inter-areal: in the non-injected area 3b or area 1.

Area Injected	Total (large, small)	Intra-areal (large, small)	Inter-areal (large, small)
Area 3b	27 (19,8)	13(9,4)	14(10,4)
Area 1	28(12,16)	16(6,10)	12(6,6)
Totals	55(31,24)	29(15,14)	26(16,10)

Table 2.

Correlation between the different variables used. Data are shown only for the 32 boutons, which formed PSD (Table 3). Mitochondrial measures were compared in 24 of the 32 boutons, which contained mitochondrion. All except PSD surface correlations were significant ($p < 0.05$).

Variables	B	M	vol	sf	sf	sf
	sf vs. vol*	sf vs. vol*	M vs. B	M vs. B	PSD vs. B	PSD vs. M
Pearson R	0.96	0.94	0.50	0.84	-0.06	0.08

* : using transformed values (² surface and ³ volume); sf: surface, vol: volume, M: mitochondrion, PSD: postsynaptic density

Table 3.

Categorization of boutons based on either light microscopy (LM) or 3D electron microscopy (EM) based grouping (data represent number of boutons). Out of the 50 boutons only 32 with clear postsynaptic density were used for detailed ultrastructural quantifications of the large and small boutons. The 7 large boutons identified by EM categorization were collected from 3 animals, 1 after an area 1 injection and 2 after area 3b injections. Note that among the 6 “giant” axonal varicosities only 1 was synaptic bouton forming postsynaptic density (PSD) and the remaining 5 were excluded from the analyses.

	Total		with PSD	
	LM	EM	LM	EM
small	24	36	16	24
large	25	13	15	7
“giant”	1	1	1	1
sum	50	50	32	32

Table 4.

Redistribution of bouton numbers after 3D-EM based grouping (3D-EM-boutons) in the small and large groups (Fig. 4D,E). Only PSD forming boutons are shown. LM-bouton: LM-based categorization. BA3bij: area 3b injection, BA1ij: area 1 injection, intra: intra-areal BDA-labeled boutons, inter: inter-areally labeled boutons.

Group	pathway	BA3bij	BA1ij	sum
Large LM-boutons	total	12	3	15
	intra	5	-	5
	inter	7	3	10
Large LM regrouped to small 3D-EM	total	8	-	8
	intra	4	-	4
	inter	4	-	4
Large 3D-EM-boutons	total	4	3	7
	intra	1	-	1
	inter	3	3	6
Small 3D-EM-boutons	total	11	13	24
	intra	7	7	14
	inter	4	6	10