Ultrastructural heterogeneity of human layer 4 excitatory synaptic boutons
in the adult temporal lobe neocortex

Rachida Yakoubi¹, Astrid Rollenhagen¹, Marec von Lehe²,³, Dorothea Miller², Bernd Walkenfort⁴, Mike
Hasenberg⁴, Kurt Sätzler⁵, Joachim HR Lübke¹,⁶,⁷

¹Institute of Neuroscience and Medicine INM-10, Research Centre Jülich GmbH, Leo-Brandt Str., 52425
Jülich, Germany; ²Department of Neurosurgery, University Hospital/Knappschaftskrankenhaus Bochum,
In der Schornau 23-25, 44892 Bochum, Germany; ³Department of Neurosurgery, Brandenburg Medical
School, Ruppiner Clinics, Fehrbelliner Str. 38, 16816 Neuruppin, Germany; ⁴Medical Research Centre,
IMCES Electron Microscopy Unit (EMU), University Hospital Essen, Hufelandstr.11, 45122 Essen,
Germany; ⁵School of Biomedical Sciences, University of Ulster, Cromore Rd., BT52 1SA, Londonderry,
UK; ⁶Department of Psychiatry, Psychotherapy and Psychosomatics, Medical Faculty/RWTH University
Hospital Aachen, Pauwelsstr. 30, 52074 Aachen, Germany; ⁷JARA Translational Brain Medicine,
Germany

Address correspondence to: Joachim Lübke
Institute of Neuroscience and Medicine INM-10
Research Centre Jülich GmbH
52425 Jülich
Phone: +49-2461-612288
Fax: +49-2461-611778
E-mail: j.luebke@fz-juelich.de
Abstract

Synapses are fundamental building blocks that control and modulate the ‘behavior’ of brain networks. How their structural composition, most notably their quantitative morphology underlies their computational properties remains rather unclear, particularly in humans. Here, excitatory synaptic boutons (SBs) in layer 4 (L4) of the temporal lobe neocortex (TLN) were quantitatively investigated.

Biopsies from epilepsy surgery were used for fine-scale and tomographic electron microscopy to generate 3D-reconstructions of SBs. Particularly, the size of active zones (AZs) and of the three functionally defined pools of synaptic vesicles (SVs) were quantified.

SBs were comparably small (~2.50 μm$^2$), with a single AZ (~0.13 μm$^2$) and preferentially established on spines. SBs had a total pool of ~1800SVs with strikingly large readily releasable (~ 20), recycling (~ 80) and resting pools (~850).

Thus, human L4 SBs may act as ‘amplifiers’ of signals from the sensory periphery and integrate, synchronize and modulate intra- and extra-cortical synaptic activity.
Introduction

The neocortex of various animal species including non-human primates (NHPs) and humans is characterized by its six-layered structure, the organization into vertical oriented functional slabs so-called cortical columns and a system of long-range horizontal axonal collaterals that connect neurons in the same (intra-laminar) and in different (trans-laminar) cortical layers of a given brain area but also with trans-regional projections to different brain regions (reviewed by Rockland and De Felipe 2018).

The human TLN representing ~17% of the total volume of the neocortex (Kiernan 2012) is regarded as a highly specialized associative brain region roughly subdivided into a superior, medial and inferior gyrus that is highly interconnected with the limbic and various sensory systems. In addition, it is well documented that the TLN represents a homotypic granular, six-layered associative neocortex comparable in its cytoarchitecture with primary sensory cortices but different to heterotypic agranular motor cortex (von Economo and Koskinas 1925; Vogt 2009; Zilles et al. 2015, Zilles and Palomero-Gallagher 2017). The growing interest in the TLN is motivated by its importance in high-order brain functions as audition, vision, memory, language processing, and various multimodal associations. Moreover, the temporal lobe is also involved in several neurological diseases most importantly as the area of origin and onset of temporal lobe epilepsy (TLE; reviewed by Allone et al. 2017; Tai et al. 2018). TLE is the most common form of refractory epilepsy characterized by recurrent, unprovoked focal seizures that may, with progressing disease, also spread to other areas of the brain. Taken together, the TLN represents an important region in the normal and pathologically altered brain in humans. However, relatively little is known about its neural (but see DeFelipe 2011; Mohan et al. 2015) and synaptic organization.

Synapses are highly specialized entities involved in the neural communication at any given network of the brain. Neocortical synapses have been recently described quantitatively in rodents and NHPs (for example: Popov and Stewart 2009; Rollenhagen et al. 2015, 2018; Bopp et al. 2017; Hsu et al. 2017; Rodriguez-Moreno et al. 2018), but less is known about these structures in humans, particularly at the presynaptic site (but see Cragg 1976; Gibson 1983; Kirkpatrick et al. 2006; Alonso-Nanclares et al. 2008; Blazquez-Llorca et al. 2013; Kay et al. 2013; Bernhardt et al. 2013; Liu and Schumann 2014). Such less coherent and comprehensive quantitative studies depend on suitable human brain tissue with highly
preserved ultrastructure which can be only guaranteed using access tissue from biopsy material from epilepsy surgery (Yakoubi et al. 2018) but not by post-mortem material.

Here, we have investigated SBs in L4 of the adult human temporo-basal lobe (Gyrus temporalis inferior). In granular sensory cortices, for example the somatosensory, visual cortex and the TLN, L4 is regarded as the main recipient layer for signals from the sensory periphery and thus represents the first station of intracortical information processing (reviewed by Sherman 2012; Clascá et al. 2016; but see Constantinople and Bruno 2013).

We took advantage of non-epileptic neocortical access tissue provided from TLE surgery, and used high-resolution fine-scale transmission and tomographic electron microscopy (EM) and subsequent computer-assisted quantitative 3D-reconstructions of synaptic structures to look for structural correlates relevant for synaptic transmission and plasticity. Since this approach is very pertinent and reproducible in humans (Yakoubi et al. 2018), we can demonstrate marked species and even layer-specific differences in the synaptic organization in the human TLN, particularly in the shape and size of the AZs and in the three functionally defined pools of SVs.

The quantitative 3D-models of SBs also provide the basis for realistic numerical and/or Monte Carlo simulations of different parameters of synaptic function, for example neurotransmitter release and diffusion at AZs that remain only partially accessible to experiment at least in humans.

Results

Density of synaptic contacts established by SBs in L4 of the human TLN

First, the density of synaptic contacts was measured since such data are rare for humans particularly for the TLN (but see Marco and DeFelipe 1997; Alonso-Nanclares et al. 2008; Finnema et al. 2016). These measurements provide the basis to further gain information regarding the synaptic organization of the neuropil, rate of connectivity as well as possible inter-individual and gender-specific differences in humans (Table 1).
Table 1: Density of synaptic contacts in L4 of TLN

| *Patients | Hu_110204♀ | Hu_110520♀ | Hu_160217♀ | Average ± SD | Hu_161118♀ | Hu_170801♀ | Hu_181120♀ | Average ± SD | Total average ± SD |
|-----------|------------|------------|------------|--------------|------------|------------|------------|--------------|-------------------|
| Total density of synaptic contacts/mm³ | 4.1*10⁶ ± 2.36*10⁶ | 1.3*10⁶ ± 0.51*10⁶ | 6.0*10⁶ ± 0.8*10⁶ | 3.80*10⁶ ± 2.36*10⁶ | 0.8*10⁶ | 0.5*10⁶ | 1.5*10⁶ | 0.93*10⁶ ± 0.51*10⁶ | 2.37*10⁶ ± 2.19*10⁶ |
| Excitatory SBs on spines (%) | 60 ± 7.55 | 60 ± 7.55 | 73.08 ± 12.73 | 64.36 | 100 | 100 | 66.66 | 88.89 ± 19.25 | 76.62 ± 18.75 |
| Excitatory SBs on shafts (%) | 33.33 ± 12.73 | 40 ± 7.55 | 15.38 ± 12.73 | 29.57 | 0 | 0 | 33.33 | 11.11 ± 19.24 | 20.34 ± 17.75 |
| Inhibitory SBs on spines (%) | 6.66 ± 3.84 | 0 ± 3.84 | 0 ± 3.84 | 2.22 | 0 | 0 | 0 | 0 ± 0 | 1.33 ± 2.98 |
| Inhibitory SBs on shafts (%) | 0 ± 3.84 | 7.69 ± 4.44 | 0 ± 4.44 | 2.56 | 0 | 0 | 0 | 0 ± 0 | 1.54 ± 3.44 |

*Patients are identified by the date of the surgery as follows: Human_year/month/date

The density of synaptic contacts averaged 3.80*10⁶ synapses/mm³ in women (n=3; ranging from 25-36 years of age) and 0.93*10⁶ synapses/mm³ in men (n=3; ranging from 33-63 years in age). The overall average was 2.37*10⁶ synapses/mm³. Strikingly, a huge inter-individual variability was found: a 1.5-4-fold and 2-3-fold difference between women and men, respectively.

In all patients, the majority of synaptic contacts counted were excitatory contacts and were found predominantly on spines of different types (76.62%) and on shafts (20.34%). The remainder was inhibitory contacts mainly established on dendritic shafts (1.54%). However, GABAergic terminals were infrequently located also on spines (1.33%).

In summary, the synaptic density in L4 of the temporo-basal lobe was ~4-fold higher in women than in men, suggesting a gender-specific difference (see discussion). But the target structure innervation pattern was relatively similar among patients regardless of their gender.

Neural organization of the human TLN

In granular cortices in rodents and higher mammals including NHPs and humans, L4 is composed of two different types of spiny neurons, the majority of which represent spiny stellate cells with a smaller fraction of star pyramidal cells (Ahmed et al. 1994; Lübke et al. 2000; Staiger et al. 2004; Egger et al. 2008; Oishi
et al. 2016). In Golgi-stained and semithin sections throughout the human TLN, L4 was distinguishable from the lower part of L2/3 and L5 by the absence of pyramidal-shaped neurons (Fig. 1A, B, Child Fig. 1) and is further characterized by a zone relatively sparse of neurons with comparably small, round to ovoid cell bodies organized in a cluster-like fashion (Fig. 1B framed area, D). At the EM level, GAP-junctional coupling between dendrites was frequently observed (Fig. 1F, G) in L4, as well as tight junctional coupling mainly between astrocytes. Interestingly, L2 was composed of pyramidal cells that were densely packed but randomly distributed throughout the neuropil in semithin sections (Fig. 1A, B) whereas L3 contained somewhat larger pyramidal neurons which in contrast to L2 were more loosely distributed (Fig. 1A-C). Layers 5a, b and 6a were composed mainly of pyramidal neurons of different shape and size (Fig. 1A, Child. Fig. 1) whereas in sublayer 6b numerous inverted or horizontally oriented pyramidal neurons are found (Fig. 1E) as also described in rodents (Tömböl et al. 1984; Marx and Feldmeyer 2013).
Figure 1: The cytoarchitecture of the human TLN

A. Low power light micrograph of a methylene blue stained semithin section through the gyrus temporalis inferior (baso-lateral region) in humans, showing the typical six-layered organization of a granular neocortex. Dashed line indicates the grey/white matter border. B. High power light micrograph through the same section shown in A. Note the different density of neurons in L2 and L3. Red frame: region of interest in L4. C. High power light micrograph through layers 2 and 3. D. Cluster-like arrangement of neurons in L4. E. Neuronal organization of L6b containing several inverted pyramidal cells marked by asterisks. Scale bars A-E 100 µm. F, G, Two examples of GAP-junctions between dendrites (de1, de2) (red frame in F) and G at higher magnification. Scale bars 0.5 µm in F, 0.2 µm in G.
Child figure 1: Golgi-Cox impregnation of the TLN

A, Overview through the six layers of the TLN. Scale bar 500 µm. B, High power micrograph of the framed area in A. Asterisks indicate spiny stellate cells and star pyramidal neurons characteristics of L4. Scale bar 100 µm. Inset: Representative example of a spiny stellate cell in L4.
Quantitative analysis of L4 synaptic complexes in the human TLN

The main goal of this study was to quantify several morphological parameters representing structural correlates of synaptic transmission and plasticity in L4 excitatory SBs in the human TLN. For this purpose, 150 SBs and 155 AZs were completely reconstructed out of five series of 70-100 ultrathin sections/series using biopsy material from TLE surgery (see Material and Methods).

EM investigation of L4 in the human TLN revealed a dense neuropil containing neuronal cell bodies, astrocytes and their fine processes, dendrites and SBs of different shape and size (Fig. 2, 3) and traversing apical dendrites of L5 pyramidal neurons (Fig. 1B) which are much thicker in diameter and were thus not sampled.

Synaptic complexes in L4 were formed by either presynaptic en passant or endterminal boutons (Fig. 2), with their prospective postsynaptic target structures, either a cell body of a neuron, or dendritic shafts of different calibers (17.7 %) or spines of distinct sizes and types (thin 58.20%; filopodial 5.70%; mushroom 7.80%; stubby 3.50% and 7.10% were not classifiable). In our samples SBs were predominantly (~82% of the total) found on dendritic spines, ~3% of which are SBs establishing either two or three synaptic contacts on the same spine and numerous contacts with either the same or different dendrites (Fig. 2B, C). Interestingly, only ~40 % of the spines in our sample contained a spine apparatus (Figs. 2B, 3A, B), a specialized form of the endoplasmic reticulum.

L4 SBs were on average small, with a mean surface area of 2.50 ± 1.78 µm², and a mean volume of 0.16 ± 0.16 µm³, respectively. SBs were oval to round with a form factor, ranging from 0.27 to 0.77 and a mean of 0.56 ± 0.09. Beside relatively large SBs (11.54 µm²; 1.09 µm³) also very small ones (0.42 µm²; 0.01 µm³) were sampled and quantified. Hence, a huge variability was observed with respect to the shape and size of SBs as indicated by the large SD, CV and variance (Table 2) regardless of their target structures. Interestingly, a high correlation between the surface area and volume of SBs was observed as indicated by the coefficient of correlation (R²; Fig. 5A).

In larger SBs, several mitochondria (range 1 to 4) of different shape and size (0.02 ± 0.03 µm³) were present, occupying ~13% of the total bouton volume (Figs. 2A, 3B, 4A), whereas smaller SBs contained no (Figs. 2A, 4A, B) or only a single mitochondrion (Figs. 2A, C, 3C). Thus a strong correlation was found
between the volume of the SBs and the volume of mitochondria (Fig. 5B), suggesting an important role of mitochondria in the function of the SB (see Discussion).

Figure 2: Innervation patterns of L4 SBs and target specificity

A, Dense innervation of endterminal SBs (b1-b8; transparent yellow) terminating on a dendrite (de; transparent blue) of ~5 μm length. Note the presence of unmyelinated axons (asterisks) isolating SB b5 from SB b6. Scale bar 1 μm. B, En passant SB (b) innervating a dendritic shaft (sh) and a spine (sp) identified by a spine apparatus (framed area). C, Large endterminal SB (b) innervating two small spines (sp1, sp2). Scale bars in B and C 0.5 μm. Note the presence of multivesicular bodies (mvb) in A and B. In all images the AZs are marked by red arrowheads. In B and C same color code as in A.
Figure 3: L4 SBs innervate different types of spines

A, Two SBs (b1, b2; yellow contour) terminating on two stubby spines (sp1, sp2; blue contour). Scale bar 0.5 µm. B, SB (b; yellow contour) on the head of a large mushroom spine (sp; blue contour). Note the presence of two mitochondria occupying a large fraction of the total volume of the SB. Here, SVs were found in closer proximity to the PreAZ. Scale bar 0.25 µm. C, SB (b; yellow contour) terminating on the head of an elongated spine (sp; blue contour) emerging from a relatively large dendritic segment (de; blue contour) containing a multivesicular body (mvb). Scale bar 0.5 µm. Note the presence of a spine apparatus (framed area) in A and B. All AZs are highlighted in transparent red.
Worth mentioning is the observation of a few degenerating SBs, characterized by their content of distorted organelles, as well as the presence of several apoptotic neurons identifiable by their dark appearance (Fig. 1), severe distortions of their cytoplasm and the presence of microglia, indicative of cell death of these neurons in our biopsy samples (not shown).

### Table 2: Quantitative analysis of various synaptic parameters

|                          | Mean ± SD  | Median | IQR | CV  | Skewness | Variance |
|--------------------------|------------|--------|-----|-----|----------|----------|
| **Synaptic boutons**     |            |        |     |     |          |          |
| Surface area ± SD (µm²)  | 2.50 ± 1.78| 2.05   | 1.67| 0.72| 1.97     | 3.24     |
| Volume ± SD (µm³)        | 0.16 ± 0.16| 0.11   | 0.12| 1.01| 2.89     | 0.03     |
| **Active zones**         |            |        |     |     |          |          |
| PreAZ surface area ± SD (µm²) | 0.13 ± 0.07 | 0.11 | 0.08 | 0.54 | 1.35     | 0.005    |
| PSD surface area ± SD  (µm²) | 0.13 ± 0.07 | 0.11 | 0.08 | 0.53 | 1.44     | 0.005    |
| **Cleft width ± SD (nm)**|            |        |     |     |          |          |
| Lateral                  | 14.11 ± 0.69| -     | -   | 0.05| 0.74     | 8.86     |
| Central                  | 16.47 ± 1.85| -     | -   | 0.11| 0.80     | 17.09    |
| **Mitochondria**         |            |        |     |     |          |          |
| Volume ± SD (µm³)        | 0.03 ± 0.04 | 0.02  | 0.02| 1.04| 3.71*    | 0.001    |
| % to the total volume    | 13.11 ± 6.20| 12.78 | 9.25| 0.47| 0.17     | 38.47    |
| **Synaptic vesicles**    |            |        |     |     |          |          |
| Total number             | 1820.64 ± 980.34| 1544.5| 1119.5| 0.54| 0.91 | 961066.59 |
| Diameter (nm)            | 19.80 ± 5.63| 18.00 | 0.28| 3.41| 2.10     | 31.69    |
| Volume (µm³)             | 0.01 ± 0.01| 0.01  | 0.01| 1.28| 3.95*    | 0.0002   |
| **Pool size of SVs**     |            |        |     |     |          |          |
| Putative RRP at p10 nm   | 20.20 ± 18.58| 17    | 27.25| 0.92| 1.11     | 345.04   |
| Putative RRP at p20 nm   | 48.59 ± 39.02| 41    | 53   | 0.80| 1.17     | 1523.14  |
| Putative RP 60-200 nm    | 382.1 ± 248.23| 313  | 376.79| 0.65| 1.41     | 61617.55 |
| Putative reserve pool >200 nm | 1251.82 ± 471.17| 541 | 471.17| 0.38| 1.70     | 87678.29 |

Summary of structural parameter measurements quantified from the detailed 3D-reconstructions of SBs (N=150) in L4 of the human TLN. Means ± SD, Median, Interquartile Range and CVs were given for each parameter investigated and all the data were pooled. For the cleft width, measurements were taken at the two lateral edges of the PreAZs and PSDs. A mean ± SD was calculated from the two values. The cleft width at the central region under the PreAZs and PSDs was also measured. *: Values with a skew > 3 indicating non-normal distributions.

### Structural composition of AZs in L4 excitatory SBs in the human TLN

The number, size and shape of the AZs are key structural determinants in synaptic transmission and plasticity. The majority (~97%) of SBs in L4 had only a single (Figs. 2A, 3) at most three AZs. Beside very
large AZs spanning the entire pre- and postsynaptic apposition zone (Fig. 2A), also quite small AZs covering only a fraction of the apposition zone were found (Figs. 2, 3). The majority of AZs (~63%) showed either a perforation in the PreAZ and PSD or both (Fig. 3B, 4D); the remainder was non-perforated (Figs. 2, 3A, C). Interestingly, 38.46% of the perforated AZs were established on spines featuring a spine apparatus.

On average, PreAZs were $0.13 \pm 0.07 \, \mu m^2$ and PSDs $0.13 \pm 0.07 \, \mu m^2$ in surface area, ranging from 0.03 to 0.39 $\mu m^2$ (PreAZs), and from 0.03 to 0.41 $\mu m^2$ (PSDs) with several AZs that were ~2-fold larger than the mean. However, as also described for other CNS synapses of similar size, a huge variability in the shape and size of the PreAZs and PSDs was observed as indicated by the SD, CV and variance (Table 2). Strikingly, only a weak correlation between the mean surface area of SBs and that of PreAZs (Fig. 4C) was observed. Interestingly, the PreAZ and PSD areas forming the AZ overlapped perfectly in size as indicated by the ratio and the correlation factors ($1.01 \pm 0.14$; Fig. 4D). Finally, only a weak correlation existed between the PreAZs surface area and the total pool of SVs (Fig. 5F) suggesting that both the PreAZ size and the total pool of SVs may be independently regulated from the size of the SBs.

Strikingly, the mean surface area of the PreAZs on spines, regardless of the spine type, was almost identical when compared to those found on dendritic shafts ($0.13 \pm 0.07 \, \mu m^2$ vs. $0.11 \pm 0.05 \, \mu m^2$) although this difference was not significant ($p=0.06$ using H-test).

The width of the synaptic cleft was $14.11 \pm 0.69 \, nm$ for the lateral, and $16.46 \pm 1.85 \, nm$ for the central region. No clear difference, e.g. the typical wide broadening of the synaptic cleft, was observed at AZs in our sample, although there is a high variability as indicated by the variance for both the lateral and central edges, respectively ($0.04 \, vs. \, 0.11$).

**Organization of the pools of SVs in L4 excitatory SBs of the human TLN**

SVs are the key structures in storing and releasing neurotransmitters, and hence play a fundamental role in synaptic transmission and in modulating short- and long synaptic plasticity. Their distribution in the terminal and organization into three distinct functional pools, namely the readily releasable (RRP), the recycling (RP) and the resting pool, regulate synaptic efficacy, strength and determine the mode and probability of release ($P_r$; uni- vs. multi-vesicular; uni- vs. multi-quantal) (Saviane and Silver 2006; Watanabe et al. 2013;
Figure 4: 3D-reconstructions showing the high variability in the size of the total pool of SVs

A, 3D-volume reconstruction of a SB (yellow outlines) innervating a dendritic shaft (sh; blue) and two spines (sp1, sp2; blue). Note the relatively large total pool of SVs (green dots); the size of the PreAZs (red) and the mitochondria (white) always associated to the pool of the SVs. B-D, 3D-volume reconstructions of individual total pools of SVs (green dots) at either non-perforated (B, C) or perforated AZs (D). Large DCVs (magenta) were frequently observed. E, Example of a mitochondrial-derived vesicle (framed area) splitting from the mitochondrion. Scale bar 0.1 µm.
In general, SVs were distributed throughout the entire terminal in ~85% of the population of SBs investigated (Figs. 2, 3A, C, 4). The remainder population of SBs was characterized by rather densely packed or ‘clustered’ SVs near the PreAZs (Fig. 3B).

Different types of SVs were found: (1) Small clear SVs with a mean diameter of 19.79 ± 5.62 nm, (2) Large clear SVs with a mean diameter of 69.74 ± 12.26 nm, (3) Mitochondrial-derived vesicles (MDVs) were rarely observed (Fig. 4E); their role still remains rather unclear. However, they might be involved in the selective transport of mitochondrial substances to lysosomes for degradation (Sen and Cox 2017; reviewed by Sugiura et al. 2014; Misgeld and Schwarz 2017) and (4) Large DCVs with an average diameter of 42.98 ± 14.45 nm. DCVs were seen to either fuse with the presynaptic membrane or intermingled with the population of the SVs throughout the SB (Fig. 4C). Considering these locations one might already deduce the possible functions of the DCVs, namely: their implication in endo- and exocytosis (Watanabe et al. 2013) and build-up of AZs by releasing Piccolo and Bassoon (Schoch and Gundelfinger 2006), or by clustering SVs at the PreAZs (Mukherjee et al. 2010, Watanabe et al. 2013). In addition various co-transmitters, such as neuropeptides, ATP, noradrenalin, and dynorphin were identified in large DCVs (Ghijsen and Leenders 2005; Zhang et al. 2011).

In our study, the average total pool of SVs was 1820.64 ± 980.34 SVs (ranging from 368 to 5053) occupying ~7% (0.01 µm³) of the total volume of SBs, although the range and the SD indicated a huge variability in total pool size (Table 2) at individual SBs. Strikingly, the total pool in the human TLN was already ~3-fold larger when compared with L4 and L5 SBs in rats (Rollenhagen et al. 2015, 2018), although only a low correlation was found between the total pool of SVs and PreAZ surface area (Fig. 5E) as well as volume of SBs (Fig. 5G) implying that the total pool of SVs were independent from the size of the SBs.

The distribution pattern of SVs made it impossible to morphologically distinguish the three functionally defined pools of SVs, except for the ‘docked’ vesicles primed to the PreAZ (reviewed by Rizzoli and Betz 2005; Denker and Rizzoli 2010; Chamberland and Tóth 2016). One method to overcome this problem is to perform a distance (perimeter) analysis that determined the exact location of each SV from the PreAZ. Thus, we assumed that the RRP was located at a distance (perimeter p) of ≤10 nm and ≤20 nm from the PreAZ representing docked and primed SVs fused to the PreAZ. The second pool, the RP, is constituted by SVs
within 60-200 nm, which maintained release on moderate (physiological) stimulation. The resting pool, consisted in all SVs further than ≥200 nm, preventing depletion upon strong or repetitive stimulations, but which under normal physiological conditions remains unused.

Using the same perimeter criteria as at human L5 SBs (Yakoubi et al. 2018), the RRP/AZ was extremely large with an average of 20.20 ± 18.57 at p10 nm and increased by nearly 2.5-fold (48.59 ± 39.02) at p20 nm. However, both pools were characterized by a large variability as indicated by the SD, CV and variance (Table 2) suggesting differences in \( P_r \), synaptic efficacy, strength and paired-pulse behavior at individual SBs. Interestingly, no correlation was found for the p10 nm and p20 nm RRP with the surface area of PreAZs (Fig. 5H) in contrast to CA1 synapses (Matz et al. 2010).

The RP/AZ was also comparably large with 382.01 SVs at 60-200 nm but also with a large variability at individual SBs. The resting pool contained on average 1438.63 SVs and showed the same variance in pool size as the RRP and RP.

However, no correlation was found between the RRP at p10 nm (Fig. 6A) and p20 nm (Fig. 6B) and the total pool of SVs minus the RRP at p10 nm and p20 nm, respectively. A weak correlation was observed for the RP (p60-p200 nm) and the total pool of SVs (Fig. 6C-E). Finally, no correlation existed for the resting (p500 nm) and the total pool of SVs (Fig. 6F).

Taken together, although small in surface area and volume, SBs in L4 of the TLN have strikingly large RRP, RPs and resting pools when compared with CNS synapses of comparable size or even much larger terminals (see Discussion), but all pools were characterized by a huge variability and were not correlated and hence independent from the SB and PreAZ size.

**EM tomography of L4 excitatory SBs in the human TLN**

High-resolution EM tomography was carried out on a sample of small to large SBs with different AZ sizes to look for the organization of SVs, in particular those of the RRP and to test the hypothesis that larger PreAZs display more primed or fused SVs than smaller ones.
Figure 5: Correlations between various structural parameters of L4 SBs

A, The surface area vs. the volume of the SBs. B, The volume of SBs vs. the volume of mitochondria. C, The surface area of SBs vs. the surface area of the PreAZs. D, The surface area of PSDs vs. the surface area of the PreAZs. E, The surface area of SBs vs. the total pool of SVs. F, The surface area of the PreAZs vs. the total pool of SVs. G, The volume of SBs vs. the total pool of SVs. H, The surface area of the PreAZs vs. p10 nm (blue dots) and p20 nm (red dots) RRsPs, respectively. *Data points were fitted by linear regression and the R² is given for each correlation.
Figure 6: Correlations between various structural parameters of L4 SBs and SVs

A, The total pool of SVs vs. the RRP at p10 nm. B, The total pool of SVs vs. the RRP at p20 nm. C, The total pool of SVs vs. the RP at p60 nm. D, The total pool of SVs vs. the RP at p100 nm. E, The total pool of SVs vs. the RP at p200 nm. F, The total pool of SVs vs. the resting pool at p500 nm.

The results of the tomography were three-fold: First, all SBs analyzed, regardless of their target structures, a dendritic shaft (Fig. 7A; Movie 1) or spine (Fig. 7B; Movie 1), contained more than one, the most seven ‘docked’ SVs and/or omega-shaped bodies, already fused (Fig. 7C-F) with the PreAZ membrane and opened to release neurotransmitter. On average $5.53 \pm 0.84$ SVs were found at individual PreAZs which was nearly 4-fold smaller when compared to the results of our quantitative perimeter analysis for the p10 nm criterion (20.20 ± 18.58 SVs; see also Table 1). Secondly, there was a tendency that larger PreAZs contained more ‘docked’ vesicles (Fig. 7A, B); providing a larger ‘docking’ area allowing the recruitment of more SVs. However, in a few cases also SBs with a smaller PreAZ were found that had the same number of ‘docked’
vesicles (Fig. 7C, E). Finally, so-called MDVs and clathrin-coated pits were clearly identified at several SBs (Figs. 4A, 7A; Movie 1).

**Figure 7: EM tomography of L4 SBs in the TLN**

A, Example of a SB (sb) terminating on a dendritic shaft (sh) with a large, perforated AZ marked by arrowheads. Asterisks indicate ‘docked’ SVs. The two frames point to the MDVs and the circle, the clathrin-coated pit. Scale bar 0.25 µm. B, Axo-spinous synapse (sb, sp) with a large non-perforated AZ (arrowheads) and an omega-shaped body (asterisk). Scale bar 0.25 µm. C, D, E, F, Four examples of high-power images of AZs where ‘docked’ SVs or omega-shaped bodies were marked by asterisks. Scale bar 0.1 µm.
The cluster analysis (CA) revealed two groups of SBs according to their structural parameters analyzed. The principal component analysis (PCA) showed two principal components (PCs) (PC1, PC2) explaining the most variance (Fig. 8A); where AZs and SV pools were the main features (parameters) that predominantly contributed to the PCs. The subsequent hierarchical cluster analysis (HCA) based on the AZs and SVs pools revealed two stable groups of SBs as shown in the generated dendrograms (Fig. 8B, C), where the dissimilarity between the two groups is indicated by the Euclidean height. In Fig. 8B, the SBs in the first group (red cluster) had large AZs with 0.14 ± 0.08 µm² for both PreAZs and PSDs surface area; whereas the SBs in the larger group (blue cluster) had smaller AZs (0.12 ± 0.06 µm²) surface areas.

The clustering according to SV pools led also to two groups of SBs, namely SBs (blue cluster) with 25.38 ± 21.09 SVs at p10 nm; 427.29 ± 288.94 SVs in the RP and 995.88 ± 696.49 SVs in the resting pool, respectively. SBs belonging to the red cluster with a smaller pool size compared to the first group: 18.69 ± 17.58 SVs at p10 nm; 368.85 ± 234.72 SVs at RP and 814.45 ± 484.73 SVs at the resting pool respectively.

Although two different clusters existed that further helped to identify subclasses of SBs according the structural parameters, excitatory L4 SBs in the TLN were relatively similar (Fig. 8C).

Thus, the CA revealed that the AZs and pools of SVs were the structural parameters that best characterized the SBs in L4 of the human TLN, clustering them into two major groups accordingly.
Figure 8: CA of PreAZs/PSDs surface area and SVs pools

A, Bar histogram showing the PCs of all structural parameters analyzed. B, C, Two dendrograms generated from the CA, identifying two groups (clusters) of L4 SBs according to the PreAZs/PSDs surface area (B) and the size of SV pools (C). The Euclidian height indicates the difference or dissimilarities between the clusters.
Astrocytic coverage of L4 SBs in the human TLN

Astrocytes by directly interacting with synapses play an important role in the induction, maintenance and termination of synaptic transmission and plasticity (Krencik et al. 2017; reviewed by Dallérac et al. 2018).

In L4 of the TLN, astrocytes and their fine processes formed a dense network within the neuropil of the TLN intermingled with neurons and synaptic complexes, composed of the SBs and dendritic shafts or spines. The majority of individual or multiple synaptic complexes (~80%) were tightly enwrapped by fine astrocytic processes physically isolating them from the surrounding neuropil and from neighboring synaptic complexes as shown in L5 of the human TLN (Yakoubi et al. 2018).

Astrocytic fingers reached as far as the synaptic cleft under the pre- and postsynaptic density (Fig. 9A).

Hence, it is most likely that fine astrocytic processes at human L4 synaptic complexes are involved in the uptake of neurotransmitter molecules in the synaptic cleft, regulating their temporal and spatial concentration and limiting intercellular crosstalk mediated by volume transmission.

Strikingly, fine astrocytic processes received direct synaptic input from SBs (Fig. 9B), as also described for L5 SBs of the human TLN (Yakoubi et al. 2018), the hippocampus and cerebellar climbing fibers (reviewed by Allen 2014; Papouin et al. 2017). On the other hand they provided direct input to postsynaptic structures as exemplified by a dendrite in Fig. 9C.

**Figure 9: Astrocytic interactions**

A, Fine astrocytic process (as) reaching as far as the synaptic cleft at the synaptic apposition zone between a spine (sp) and a SB (b). B, C, Direct synaptic contact established between an astrocytic finger (as) with a SB (b) in B, and a dendrite (de) in C. Note the presence of SVs in the dendrite in C. Scale bars in A-C 0.5 µm. In all images the AZs are indicated by red arrowheads in A-C.
Discussion

The present study is the first comprehensive and coherent structural study of L4 excitatory SBs in the temporo-basal human TLN using high-end fine-scale EM and tomography. Although SBs in any given region of the brain are composed of nearly the same structural subelements, it is their individual and specific composition that makes them unique entities, perfectly adapted to their function in the microcircuit in which they are embedded.

Here we demonstrated marked species and layer-specific disparities of SBs of the human TLN, in particular in the shape and size of PreAZs and PSDs, and even more importantly in the size of the three functionally distinct pools of SVs. These pools in L4 SBs of the human TLN are extremely large by 2-3-fold larger when compared with various animal species and SBs of comparable or even larger size, in particular the RRP and RP.

Based on our findings L4 SBs are strong, efficient and reliable in synaptic transmission. On the other hand, the large variability in the shape and size of AZs and that of the three pools of SVs at individual SBs also implies a strong modulation of short-term plasticity (see Feldmeyer et al. 1999; Seeman et al. 2018).

Relevance and implications of the density of synaptic contacts measurements

Synaptic density measurement can be a useful tool to not only describe the synaptic organization of a particular area, nuclei and even layers in different brain regions, but also the degree of connectivity underlying the computational properties of a given brain area.

Meanwhile several studies in various animal species and brain regions included such an analysis (for example: cat: Keller et al. 1992; monkey: Beaulieu et al. 1992; Peters et al. 2008; mouse: Merchán-Pérez et al. 2009; Bopp et al. 2017; reviewed by DeFelipe 1997; rat: Anton-Sanchez et al. 2014; monkey and mouse: Hsu et al. 2017); but data for synaptic density measurements in humans are comparably rare (Davies et al. 1987; Scheff and Price 1993; Blazquez-Ilorca et al. 2013; Finnema et al. 2016), in particular for the TLN (Marco and DeFelipe 1997; DeFelipe et al. 1999; Tang et al. 2001; DeFelipe et al. 2002; Alonso-Nanclares et al. 2008). Strikingly, a huge difference in the mean density of synaptic contacts was found between our study (2.37*10^6 ± 2.19*10^6) and the existing data ranging from 9.13 ± 0.63*10^8 (Alonso-Nanclares et al. 2008).
This disparities may be attributed to the age, gender, layers and regions investigated and methods used (DeFelipe et al. 1999).

In line with DeFelipe and co-workers (Alonso-Nanclares et al. 2008) a marked gender-specific difference was found, however with a higher density of synaptic contacts in women, in contrast to the above cited publication that reported higher values in men; while in other NHPs, no gender differences existed (monkey, Peters et al. 2008). Our findings may represent the structural correlate to functional gender specific differences in the brain, for example that women perform better at languages (Joseph 2000; Kimura 2000; Alonso-Nanclares et al. 2008); since the TLN is also involved in the memory and language processing and comprehension. Further density measurements are required to demonstrate similarities or differences between the three temporal gyri in the human TL or in comparison to NHPs.

In conclusion the TLN exhibited a comparably high density of synaptic contacts (see also Finnema et al. 2016), which together with the large size of the AZs and the three SVs pools demonstrated in the present study imply a high intrinsic and extrinsic connectivity of the TLN, thus shaping the network properties in which the TL is embedded.

**Shape and size of AZs at excitatory L4 SBs in the human TLN**

One of the most important structural parameter determining for example $P_r$ is the shape and size of the AZs (Matz et al. 2010; Südhof 2012; Holderith et al. 2012; Wilhelm et al. 2014). The majority of L4 SBs in the human TLN had only a single AZ as also demonstrated for other cortical SBs of similar size (Marrone et al. 2005; Nava et al. 2014; Rollenhagen et al. 2015, 2018; Bopp et al. 2017; Hsu et al. 2017). Strikingly, the surface area of AZs in L4 SBs was on average 0.13 $\mu$m² although these boutons are among the smallest in the neocortex. However, they were comparable in AZ size with L4 and L5 SBs in rats (Rollenhagen et al. 2015, 2018), but were ~2 to 3-fold larger than those in mouse and NHP visual, motor and somatosensory neocortex (Bopp et al 2017; Hsu et al. 2017). Surprisingly, AZs were even larger than in much bigger CNS terminals such as the Calyx of Held (Spirou et al. 1998; Sätzler et al. 2002; Wimmer et al. 2006) the cerebellar (Xu-Friedman and Regehr 2003); and hippocampal mossy fiber boutons (MFBs; Rollenhagen et
It has to be noted though that a large variability in both shape and size was observed in all SBs where quantitative data are available.

This variability in AZ size may partially contribute to differences in the mode of release (uni- or multivesicular; uni- or multiquantal release) and quantal size, the size of the RRP and $P_r$, as shown for other CNS synapses (Matz et al. 2010; Freche et al. 2011; Holderith et al. 2012; reviewed by Xu-Friedman and Regehr 2004). In addition, ~63% of AZs showed perforations in their PreAZ and/or PSD, which is higher than reported for L4 SBs (~35%) and comparable with values in L5 (~60%) in rats. A strong correlation between AZs surface area, perforated PSDs and the number of ‘docked’ and resting pool SVs was reported for rat neocortex (Nava et al. 2014; Rollenhagen et al. 2015, 2018) and is also supported by the findings in this study. It has to be noted that a large proportion of the AZs on spines nearly occupied two-third or even the entire pre- and postsynaptic apposition zone, suggesting that excitatory synaptic transmission is highly efficient at these structures by increasing the docking area for primed and ‘docked’ SVs which is further supported by our EM tomography experiments (see Fig.7). Interestingly, only a weak correlation between the PreAZ surface area with that of the bouton was found in human and cortical SBs in rat (Rollenhagen et al. 2015, 2018), implying that the size of the AZs is an independent structural parameter and may be regulated in an activity-dependent manner as shown for hippocampal SBs in the CA1 subregion (Matz et al. 2010; Holderith et al. 2012).

Only a weak correlation was found between the surface area of the PreAZ and the total pool of SVs, with no correlation for the p10 nm and p20 nm RRP, respectively. A slight tendency was found that SBs with larger PreAZs contained more ‘docked’ vesicles which is in contrast to our quantitative analysis concerning the RRP, but in good agreement with our tomography experiments and studies in the hippocampal CA1 region where a direct correlation between the size of the AZ and the number of SVs in the RRP together with an increase in $P_r$ was demonstrated (Matz et al. 2010 Holderith et al. 2012). However, our sample size with EM tomography was too small to substantiate this correlation for PreAZs in L4 SBs of the TLN.

Altogether, the relatively large size, perfect overlap and high number of perforated PreAZs and PSDs at L4 SBs in the human TLN may partially contribute to a high $P_r$, and thus reliable synaptic transmission. On
the other hand, the large variability in AZ size at individual SBs may play a role in the modulation of synaptic plasticity and paired-pulse behavior at individual SBs.

**Size of the three pools of SVs**

During prolonged and intense activity, synaptic transmission could be modulated in various ways depending on the availability of SVs and on their recycling rates. Hence, the size of the RRP critically determines synaptic efficacy, strength and plasticity as described for various CNS synapses (Rosenmund and Stevens 1996; Schikorski and Stevens 2001; Rizzoli and Betz 2004; Schikorski 2014; Watanabe et al. 2014; Rollenhagen et al. 2018; reviewed by Rizzoli and Betz 2005; Neher 2015; Chamberland and Tóth 2016).

Although smallest in size around neocortical SBs, L4 SBs in human TLN had a total pool size of ~1800 SVs/AZ; comparable to L5 terminals in the human TLN (~1500 SVs/AZ, Yakoubi et al. 2018), but nearly more than 3-fold (~550 SVs/AZ; Rollenhagen et al. 2015), and ~2-fold (~750 SVs/AZ; Rollenhagen et al. 2018) larger than their counterparts in L4 and L5 in rats. Comparison with even giant CNS terminals, for example adult MFBs (~20-fold larger in size), revealed a total pool size of ~850 SVs/AZ (Rollenhagen et al. 2007), ~600 SVs/AZ at cerebellar MFBs (Saviane and Silver 2006), and even a nearly 12-fold larger total pool (~125 SVs/AZ) when compared to that of the largest CNS terminals, the Calyx of Held (Sätzler et al. 2002).

The already large size of the total pool of SVs in L4 SBs in the human TLN also predict comparably large RRP s and RPs. Indeed, the putative RRP was on average 20.20 ± 18.57 (p10 nm) and doubled to 48.59 ± 39.02 (p20 nm) SVs/AZ, ~5-fold larger than those in rats (p10 nm 3.9 ± 3.4, p20 nm 11.56 ± 4.2; Rollenhagen et al. 2018), and 8-10-fold larger than that in L4 SBs in rats (p10 nm 2.0 ± 2.6, p20 nm 6.3 ± 6.4; Rollenhagen et al. 2015), respectively. Comparison with even larger CNS synaptic terminals revealed a more than 12-fold and 8-fold difference (hippocampal MFBs p10 nm 1.6 ± 1.5, p20 nm 6.2 ± 4.1; Rollenhagen et al. 2007) and Calyx of Held (p10 nm 1.9 ± 2.0, p20 nm 4.8 ± 3.8; Sätzler et al. 2002). Our estimates of the size of the RRP is even more substantiated and supported by our EM tomography. Although the number of ‘docked’ SVs and omega-shaped bodies was ~4-fold smaller than the average size of the p10
nm RRP this can be explained by the inclusion of SVs that are not docked but within 10 nm from the PreAZ, whereas only ‘docked’ SVs were counted using EM tomography.

Hence SVs in the RRP are rapidly available to sensory stimulation and execution of complex behaviors as well as up-states (Zhou and Fuster 1996; Sanchez-Vives and McCormick 2000; Sakata and Harris 2009).

This notion is even more supported by the size of the putative RP/AZ which was ~380 SVs at human L4 SBs, ~130 and~200 SVs in rat L4 and L5 SBs, respectively, ~3700 SVs for adult rat MFBs, but nearly 4-fold larger than that reported for the rat Calyx of Held (~60 vesicles). Finally, the resting pool of SVs is large enough to rapidly replenish the RRP and RP and thus guarantee only a partial depletion even at repetitive high-frequency stimulation.

Taken together, the comparably large size of AZs and that of the three pools of SVs determines and provide the basis for high reliability in synaptic transmission, efficacy and strength. The marked differences in AZ and SV pool sizes between individual SBs may underlie rapid changes in the computational properties of single neurons or networks during Up- and Down states in behavior.

Other important structural components of synaptic complexes in L4 of the human TLN

It has to be noted that in contrast to rat and mouse neocortex, the majority of L4 SBs (~85%) in the human TLN were established on spines of different types. In addition, not all shaft synapses were GABAergic and in turn not all SBs terminating on spines were glutamatergic, although such contacts were only rarely observed (see also Kwon et al. 2018). Approximately half of all spines in L4 of the human TLN contained a spine apparatus, a highly specialized derivate of the endoplasmic reticulum, involved in spine motility and the stabilization of the SB and its target structure at the pre- and postsynaptic apposition zone. The high abundance of a spine apparatus confirms and support findings in rat L4 and L5 (Rollenhagen et al. 2015, 2018) and human L5 synaptic complexes in the TLN (~65%; Yakoubi et al. 2018), but differs substantially to hippocampal CA1 synapses where only a smaller fraction (~20%) of spines contained these structural subelements (Martone et al. 1996; Špaček and Harris 1997; Deller et al. 2003). It has been demonstrated that the abundance of a spine apparatus partially contribute in modulating short- and long-term potentiation...
by stabilizing the axo-spinous complex during the initial high-frequency stimulation (Holtmaat et al. 2005; Umeda et al. 2005).

Mitochondria are important structural components present in all CNS nerve terminals but with marked difference in their numbers. At L4 SBs in the human TLN they are often organized in clusters associated with the pool of SVs, in line with observations at human L5 SBs in the TLN (Yakoubi et al. 2018) and several other CNS synapses in various animal species (for example see Rowland et al. 2000; Wimmer et al. 2006; Rollenhagen et al. 2007, 2015; Smith et al. 2016). Mitochondria are reported to be highly mobile (Mironov 2006; Mironov and Symonchuk 2006), act as internal calcium stores (Pozzan et al. 2000; Rizzuto et al. 2000) and hence regulate internal Ca\(^{2+}\) levels in nerve terminals (Perkins et al. 2010). More importantly, they are involved in the mobilization of SVs from the resting pool (Verstreken et al. 2005; Perkins et al. 2010; Smith et al. 2016). In L4 SBs of the human TLN mitochondria contribute to ~12% of the total volume of the boutons suggesting a strong contribution in the induction of several signal cascades, for example in the priming and docking process, relying on the rapid availability of Ca\(^{2+}\) in the SB.

Finally, the majority of synaptic complexes (~85%) in L4 of the human TLN were tightly enwrapped by fine astrocytic processes reaching as far as the synaptic cleft. This is in line with findings at other small-sized CNS synapses (Xu-Friedman et al. 2001; Rollenhagen et al. 2015, 2018; Yakoubi et al. 2018), but in marked contrast to the hippocampal MFB (Rollenhagen et al. 2007), the calyx of Held-principal neuron synapse (Müller et al. 2009) and hippocampal CA1 synapses (Ventura and Harris 1999). There, only ~50% were directly found at the synaptic interface (Ventura and Harris 1999) suggesting that hippocampal astrocytes do not uniformly sample glutamate at the synaptic cleft. At hippocampal MFBs and the calyx of Held fine astrocytic processes were never seen to reach as far as the synaptic cleft. It has been demonstrated that at large CNS synapses glutamate spillover, synaptic cross talk and a switch from asynchronous to synchronous release upon repetitive stimulation occurred at these synapses (von Gersdorff and Borst 2002; Hallermann et al. 2003). Astrocytes at L4 synaptic complexes thus act as physical barriers to neurotransmitter diffusion thereby preventing spillover of released glutamate by active take-up and removal of glutamate. In addition they terminate synaptic transmission and may thus, speed-up the recovery from receptor desensitization (Danbolt 2001; Oliet et al. 2004). Both mechanisms allow the precise spatial and
temporal regulation of the neurotransmitter concentration in the synaptic cleft (Anderson and Swanson 2000).

Furthermore, astrocytes release glutamate or GABA (Le Meur et al. 2012) through vesicular exocytosis, which can also regulate synaptic transmission through activation of pre- and postsynaptic receptors (Haydon and Carmignoto 2006). Finally, astrocytes are crucial for the induction and control of spike-time dependent depression (t-LTD) at neocortical synapses by gradually increasing their Ca$^{2+}$ signaling during the induction of t-LTD (Min and Nevian 2012).

Thus it is most likely that astrocytes at L4 excitatory SBs in human TLN govern synaptic transmission and plasticity by the temporal and spatial modulation of the glutamate concentration profile.

**Functional relevance**

In general, L4 is regarded as the main input layer for thalamocortical afferents from the respective thalamic relay nuclei (Ahmed et al. 1994; Rodriguez-Moreno et al. 2018, reviewed by Clascà et al. 2016, but see Constantinople and Bruno 2013). Thus, L4 represent the first station of intracortical information processing from peripheral sensory signals. From L4, these signals are then transferred via trans-laminar connections within a cortical column and via trans-columnar axons to adjacent columns or even trans-regional to other cortical regions (reviewed by Rockland and De Felipe 2018).

In rat and mouse neocortex, and recently in humans it has been elegantly shown by paired recordings that L4-L4 excitatory synaptic connections are characterized by a comparably high synaptic efficacy and strength as indicated by their high average EPSP amplitudes (rat barrel cortex 1.0 to 1.6 mV, mouse primary visual cortex 0.54 mV, human L4 0.95 mV), low coefficient of variations (<0.4) and low failure rates (<5%) indicative for highly reliable synaptic transmission (rat: Feldmeyer et al. 1999; mouse and human: Seeman et al. 2018) when compared with excitatory connections in other cortical layers (reviewed by Lübke and Feldmeyer, 2007). Some of these L4-L4 connections in rat somatosensory cortex are so strong that high-frequency trains of postsynaptic action potentials can be evoked causally related to the high abundance of NMDA-receptors contributing with ~40% to the overall EPSP amplitude (Feldmeyer et al. 1999;
Rollenhagen et al. (2012). In addition, L4-L4 excitatory synaptic connections show a high degree of bidirectional coupling (~30%) resulting in recurrent excitation or feed-back inhibition.

Finally, rat L4 spiny neurons are highly interconnected with ~200 other excitatory spiny neurons within a ‘barrel’ column: in turn ~300-400 L4 spiny neurons converge onto a single L2/3 and L5 pyramidal neuron (Lübke et al. 2003). This intracolumnar connectivity is not only a major determinant for reliable signal transduction in L4, acting as ‘feed-back amplifiers’ even for weak signals from the sensory periphery, but also for the safe and reliable distribution of signals to other neurons located in different layers within the cortical column with which L4 neurons are interconnected.

Assuming such a scenario described above exists in L4 of the human TLN, several structural parameters contribute to its function as an important associational area involved in the induction, maintenance and regulation of various computations underlying perception, executive control, learning and memory in which the TLN plays an important role. Hence several structural subelements may contribute to reliable signal transduction: The shape and size of AZs and the large number of SVs in the RRP and RP implying a high $P_r$ underlying high synaptic efficacy and strength that contribute to feed-back amplification of even weak sensory signals, and in addition may also enhance TL intracortical information processing.

The astrocytic coverage preventing glutamate spillover further guarantees a direct control and sharpening of the transmitted signals. On the other hand, the large variability in AZ size and the three pools of SVs may be involved in the sorting, modulation, and further discrimination of intrinsic and extrinsic signals by neurons in the TLN. Together, all these characteristics ensure the proper wiring and firing of neurons in L4 of the human TLN, to accomplish its function as an input-recipient layer and help to explain information processing from incoming signals of the sensory periphery, within the TLN and from brain regions with which the TLN is interconnected.

Material and Methods

Human neocortical tissue processing for EM

Biopsy material was obtained from three male and three female patients (25-63 years in age, see Table 3) who suffered from drug-resistant TLE and underwent surgery to control the seizures. The consent of the
patients was obtained and all experimental procedures were approved by the Ethical Committees of the Rheinische Friedrich-Wilhelms-University/University Hospital Bonn (ethic votum of the Medical Faculty to Prof. Dr. med. Johannes Schramm and Prof. Dr. rer. nat. Joachim Lübke, Nr. 146/11), and the University of Bochum (ethic votum of the Medical Faculty to PD Dr. med. Marec von Lehe and Prof. Dr. rer. nat. Joachim Lübke, Reg. No. 5190-14-15; ethic votum of the Medical Faculty to Dr. med. Dorothea Miller and Prof. Dr. rer. nat. Joachim Lübke, Reg. No. 17-6199-BR), and the EU directive (2015/565/EC and 2015/566/EC) concerning working with human tissue.

Table 3: Summary of patient data

| Patient identity | Gender | Age (years) | Age at epilepsy onset (years) | Histopathological result | Antiepileptic drugs (pre-op) | Reconstructed SBs |
|------------------|--------|-------------|-------------------------------|--------------------------|-----------------------------|-------------------|
| Hu_110204 VII    | Female | 36          | 4                             | GGL                      | LTG, LEV                    | 53                |
| Hu_110520 V      | Female | 25          | 12                            | AHS                      | LTG                         | 25                |
| Hu_160217 III    | Female | 25          | 23                            | GGL                      | Zebinix, LEV                 | 25                |
| Hu_161118 IV     | Male   | 33          | 5                             | Gliosis                  | LEV, CBZ                    | 25                |
| Hu_170801 I      | Male   | 63          | 24                            | AHS                      | LEV, LTG, CBZ               | 22                |
| Hu_181120        | Male   | 49          | 36                            | AHS                      | Vimpat, ZNS                 | /                 |

AHS: Ammon's horn sclerosis; GGL: Ganglioglioma; LEV: Levetiracetam; LTG: Lamotrigine; ZNS: Zonisamide; CBZ: Carbamazepine

During surgery, blocks of neocortical access tissue from the temporo-basal regions of the inferior temporal gyrus (Fig. 10) were taken far from the epileptic focus and may thus be regarded as non-affected (non-epileptic) tissue as routinely monitored by preoperative electrophysiology and magnetic resonance imaging (MRI). Other evidence that confirms the ‘normality’ of biopsies and rules out the effect of disease and treatment is the homogeneity of synaptic parameters analyzed among patients as shown by the boxplots (Fig. 11). This has also been demonstrated by other recent structural and functional studies using the same experimental approach (Testa-Silva et al. 2014; Mohan et al. 2015; Molnár et al. 2016; Seeman et al. 2018; Yakoubi et al. 2018).

After their removal, biopsy samples of the TLN were immediately immersion-fixed in ice-cold 4% paraformaldehyde (PFA) and 2.5% glutaraldehyde (GA) in 0.1 M phosphate buffer (PB, pH 7.4) for 24 - 48 hours (hrs) at 4°C. Vibratome sections (150 -200 µm in thickness, VT1000S, Leica Microsystems GmbH, Wetzlar, Germany) were cut in the frontal (coronal) plane through the human temporo-basal neocortex and
post-fixed for 30 to 60 min in 0.5 or 1% osmium tetroxide (Sigma, Munich, Germany) diluted in PB-buffered sucrose (300 mOsm, pH 7.4) at room temperature in the dark. After visual inspection and thorough washing in PB they were dehydrated in a series of ethanol starting at 20% to absolute ethanol followed by a brief incubation in propylene oxide (twice 2 min; Fluka, Neu-Ulm, Germany). Sections were then transferred into a mixture of propylene oxide and Durcupan™ resin (2:1, 1:1 for 1hr each; Fluka, Neu-Ulm, Germany) and stored overnight in pure resin. The next day, sections were flat-embedded on coated glass slides in fresh Durcupan™, coverslipped and polymerized at 60°C for 2 days.

**Figure 10: Identification of the ROI in the human TLN**

A, Lateral view of the human right cerebral hemisphere. The areas highlighted in transparent green represents the TL. The depth of the sulci are colored in red. The arrowheads indicate the ROI in the inferior temporal gyrus. B, Midsagittal and oblique view of the human right cerebral hemisphere. Color code as in A. Brown represents the occipital lobe and purple the parahippocampal region respectively. The red circle indicates the ROI. Figures A and B were retrieved and modified from [https://en.wikipedia.org/wiki/Inferior_temporal_gyrus#/media/File:TempCapts.png](https://en.wikipedia.org/wiki/Inferior_temporal_gyrus#/media/File:TempCapts.png) under the following license: [https://en.wikipedia.org/wiki/Creative_Commons](https://en.wikipedia.org/wiki/Creative_Commons). C, Postoperative fMRI after the corresponding epilepsy surgery (selective amygdalohippocampectomy). The sampling site of the biopsy material is circled in red, representing the region between the inferior temporal gyrus and the fusiform gyrus. Abbreviations: FG: fusiform gyrus, Hippo: hippocampus, ITG: inferior temporal gyrus, LV: lateral ventricle, MTG: middle temporal gyrus, STG: superior temporal gyrus.
Figure 11: Boxplots of various structural parameters

Data distributions for each patient are indicated by the medians (horizontal bars), IQRs (framed areas), minimum and maximum (vertical lines) for the distribution of: A, Surface area of SBs; B, Volume of SBs; C, PreAZ surface area; D, PSD surface area; E, Volume of mitochondria; F, total pool of SVs. Note that most structural parameters are not significantly different.
Tissue blocks were examined under the light microscope (LM) to determine the region of interest (ROI).

Semithin sections were cut with a Leica UltracutS ultramicrotome (Leica Microsystems, Vienna, Austria), with a Histo-Diamond knife (Fa. Diatome, Nidau, Switzerland) stained with methylene-blue to identify the cortical layers, examined and documented with LM using a motorized Olympus BX61 microscope equipped with the Olympus CellSense analysis software (Olympus GmbH, Hamburg, Germany). Then, serial (70-100) ultrathin sections (50 ± 5 nm thickness) were cut through L4. ROIs within a series, containing well-preserved structures, were photographed at 8000x with a Zeiss Libra 120 (Fa. Zeiss, Oberkochen, Germany) equipped with a Proscan 2K digital camera (Fa. Tröndle, Moorenweis, Germany) using the SIS Multi Images Acquisition software (Olympus Soft Imaging System, Münster, Germany). EM images were then edited using Adobe Photoshop™ and Adobe Illustrator™ software for publication.

**Golgi-Cox impregnation of biopsy material**

Four tissue blocks were processed with the Golgi-Cox impregnation technique using the commercially available Hito Golgi-Cox OptimStain™ kit (Hitobiotec Corp, Kingsport, TE, USA). After removal of the biopsy samples, tissue were briefly rinsed twice in double distilled water (dd H₂O), and then transferred into the impregnation solution overnight at room temperature. The next day, samples were incubated in a fresh impregnation solution and stored for 14 days in the dark at room temperature. Sections were then transferred in solution 3 in the dark at room temperature for one day. Thereafter, sections were placed into a fresh solution 3 in the dark at room temperature for 6 additional days. Then, solution 3 was exchanged and samples were stored at 4° in the dark overnight. Tissue blocks were embedded in 5% Agarose (Carl Roth, Karlsruhe, Germany) diluted in dd H₂O, and sectioned with a vibratome in the coronal plane at 100-250 μm thickness and then transferred to dd H₂O. After careful removal of the agarose, free-floating sections were incubated into solution 3 for 2-3 min in the dark at room temperature, and right after placed into dd H₂O, washed several times and stored overnight. The next day, sections were put into a mixture of solutions 4 and 5 for 10 min at room temperature. Afterwards, they were rinsed twice in dd H₂O for 4 min each, and dehydrated in 50%, 70% and 95% ethanol for 5 min each, then transferred into absolute ethanol (3x5 min), defatted in xylene and finally embedded in Eukitt™, (Sigma-Aldrich, Taufkirchen, Germany) coverslipped
and air-dried. Finally, sections were examined and imaged with an Olympus BX 61 light microscope equipped with the CellSense software package (Olympus, Hamburg, Germany).

Stereological estimation of the density of synaptic contacts

The density of synaptic contacts in a given volume is a valuable parameter to assess the structural and functional changes in the brain, which are linked to the age, pathological or experimental conditions (Rakic et al. 1994; DeFelipe et al. 1999). The density of synaptic contacts was unbiasedly estimated in six patients (see Table 3) using the physical dissector technique (Mayhew 1996; Fiala and Harris 2001) by counting the synaptic complexes in a virtual volume generated by two adjacent ultrathin sections i.e. the dissector: the reference section and the look-up section (Sterio 1984; Kaplan et al. 2012). Here, counting was performed using FIJI (Schindelin et al. 2012; https://fiji.sc) on a stack of 20 aligned serial electron micrographs for each patient. An unbiased counting frame was first set and synaptic contacts to be considered (counted) are the one present in the reference section only, and meeting the following criteria: presence of PreAZ and a prominent or thin PSD separated by a synaptic cleft and SVs in the presynaptic terminal. Care was taken to distinguish between excitatory and inhibitory synaptic contacts, as well as the postsynaptic target structures (dendritic spines or shafts). Finally the density of synaptic contacts ($N_v$) per 1 mm$^3$ (see Table 1) was calculated using the formula below:

$$N_v = \frac{\sum d Q_d}{\sum d V_d}$$

where $Q_d$ is the number of synaptic contacts per dissector and $V_d$ is the volume of the dissector given by: Number of dissectors x frame area x section thickness.

3D-volume reconstructions and quantitative analysis of L4 SBs

All electron micrographs composing each series were imported, stacked, and aligned in the reconstruction software OpenCAR (Contour Alignment Reconstruction; for details see Sätzler et al. 2002). Synaptic structures of interest were outlined on the outer edge of their membranes throughout the series. 3D-volume reconstructions were then generated and the following structural parameters were analyzed: 1) surface area
and volume of SBs; 2) volume of mitochondria; 3) surface area of the PreAZs and PSDs; 4) number and diameter of clear synaptic and DCVs, and 5) total pool of SVs and the RRP, RP and resting pool.

The PreAZ surface area was measured by extraction from that of the presynaptic terminal membrane. The size of the PSD was the perimeter ratio between the outlines of the PSD to that of the synaptic contact. Synaptic cleft width measurement was performed only on synaptic contacts cut perpendicular to the AZ. The distance between the outer edge of the pre- and postsynaptic membranes at the center of the synaptic contact and at the two lateral edges was measured and averaged for each synaptic contact. All SVs were marked throughout each SB and their diameters were individually measured. To determine the distribution profile of the SVs, the minimal distance between each SV membrane to the contour lines of the PreAZ was measured throughout the SB in every single image of the series. Large DCVs were only counted in the image where they appeared largest (for details see Yakoubi et al. 2018).

No correction for tissue shrinkage was performed. Recently it has been shown by high pressure freezing, cryo-substitution and subsequent EM that no significant differences in quantitative parameters of synaptic structures as measured here were found when compared with conventionally embedded electron microscopic material (Korogod et al. 2015).

**Focused ion beam scanning electron microscopy**

In this study FIB-SEM was used on L4 of the human TLN to investigate the dynamic changes of the neuropil through a large z-dimension (Movie 2).

Immediately after explantation, one additional neocortical access tissue sample from a female patient (63 years in age) of the Gyrus temporalis inferior was immersion-fixed in an ice-cold mixture of phosphate-buffered 4% PFA and 2.5% GA for 4hr. Subsequently, the samples were post-fixed overnight in 0.15M cacodylate buffer (CB) + 2% PFA, 2.5% GA and 2mM CaCl₂ before they were embedded in 4% Agar-Agar dissolved in water. After removing access Agar-Agar, vibratome sections of 150 µm thickness were cut (VT1000S, Leica Microsystems GmbH, Wetzlar, Germany) in the frontal (coronal) plane through the human TLN. Sections were collected in multi-well plates in 0.3M CB + 4 mM CaCl₂ and thoroughly washed (5x 3 min) with 0.15M CB + 2mM CaCl₂. Thereafter, sections were incubated in 0.15M CB + 1.5% potassium...
hexocyanoferrate (II), 2% osmium tetroxide and 2mM CaCl₂ for 1 hr on ice, in the dark. After washing (5x 3 min) with deionized water (“MilliQ”, Merck Millipore, Burlington, Massachusetts, USA), sections were placed in an aqueous 1% thiocarbamoylhydrazide solution for 20 min followed by another washing step with deionized water (5x 3 min). This was followed by another treatment with an aqueous 2% osmium tetroxide solution for additional 30 min at room temperature, in the dark and washing with deionized water (5x 3 min). Block contrasting was conducted with a filtered, aqueous 1% uranyl acetate solution, overnight at 4°C, in the dark. On the next day, samples were washed with deionized water (5x 3 min) and stained with lead aspartate (20 mmol lead nitrate in a 30 mmol L-aspartic acid solution, pH 5.5) for 30 min at 60°C. After thorough washing with deionized water (3x 5 min), sections were dehydrated through an ascending series of ice-cold, aqueous ethanol dilutions (30%, 50%, 70%, 90%, 100%, each 5 min, 2x 100%, anhydrous, each 10 min) before they were transferred into propylene oxide (2x 10 min). Finally, the samples were infiltrated with an ascending series of Durcupan ACM (Sigma-Aldrich) in propylene oxide (2:1; 3:1, each for 1 hr and pure Durcupan ACM, overnight) before the sections were flat-embedded between 2 overhead projector foils, which in turn were placed between 2 microscopic glass slides and polymerized at 60°C for two days.

For the quantitative analysis of L4 synaptic boutons 3D-volume reconstructions were made based on z-stacks obtained using focused ion beam (FIB) scanning electron microscopy (SEM). Based on the overall appearance of the sample, an area of interest was trimmed out of a flat-embedded section, using a 4 mm biopsy puncher, which was then glued onto a pre-polymerized resin block. Excess resin was removed around the tissue using a histology diamond knife on an ultramicrotome (UC7, Leica Microsystems GmbH). The tissue sample was removed from the resin block with a razor blade and was then glued onto a SEM aluminum specimen stub using colloidal silver paste. The sample was dried in a vacuum chamber overnight, then sputter-coated with platinum/palladium for 15 s and finally placed into the FIB-SEM (Crossbeam© 540, Carl Zeiss, Oberkochen, Germany) for 3D analysis.

A trench was milled with the FIB at 30 kV/30 nA, polishing of the surface was performed at 30 kV/3 nA and fine milling for data acquisition was performed at 30 kV/7 nA. The cross-section surface was imaged with an electron energy of 2 keV and an electron beam current of 500 pA using an in-column energy-selective backscatter electron detector. The dwell time was 10 µs with line average 1. The pixel size in the XY-plane...
was 10 nm and the slice thickness (Z-direction) was 50 nm yielding a voxel size of 10 nm x 10 nm x 50 nm. The image acquisition software Atlas 3D (Ver. 5.2.0.125, ZEISS, Oberkochen, Germany) allowed the automated collection of 3D SEM datasets using automated correction algorithms for drift, focus and astigmatism (Movie 2). The advantage in using the FIB-SEM technique is three-fold: 1) a much higher throughput of different tissue samples at once; 2) definition of a much a larger region of interest per sample and 3) increase of the z-dimensions of the individual samples. However, the disadvantage of this method is still the weaker resolution of single SVs compared to TEM. This approach, together with TEM, will be used in future studies for further image processing, 3D-volume reconstructions and subsequent data analysis.

Movie 2: FIB-SEM sequential movie through L4 of the human TLN
Note the dynamic changes in the shape and size of dendritic and synaptic structures through the z-stack (250 single images)

EM tomography of L4 SBs in the TLN
EM tomography was carried out on 200-300 nm thick sections cut from blocks prepared for ultrathin sectioning as described above. Sections were mounted on either pioloform-coated line or slot copper grids (Plano, Wetzlar, Germany) and were counterstained with uranyl acetate and lead citrate following a slightly modified staining protocol as described by Reynolds (1963). Subsequently, sections were examined with a JEOL JEM 1400Plus, operating at 120 kV and equipped with a 4096x4096 pixels CMOS camera (TemCam-F416, TVIPS, Gauting, Germany). Tilt series were acquired automatically over an angular range of -60° to +60° at 1° degree increments using Serial EM (Ver. 3.58; Mastronarde 2005). Stack alignment and reconstruction by filtered backprojection were carried out using the software package iMOD (Ver. 4.9.7; Kremer et al. 1996). Final reconstructions were ultimately filtered using a median filter with a window size of 3 pixels.
CA of excitatory SBs in L4 of the TLN

CA was performed based on the structural parameters investigated (see Table 2), to further identify different groups i.e. types of SBs by running a CA using MATLAB and Statistics Toolbox Release 2016b (The MathWorks, Inc., Natick, MA, USA; for details see Yakoubi et al. 2018). Then a zero-mean normalization was performed as the parameters had different units. This was followed by a PCA to reduce our large dataset to a smaller set of uncorrelated variables called PCs, but still containing most of the information in the original dataset. Subsequently, we performed a HCA on the simplified dataset composed of the PCs, as the original data were not labeled (Fig. 8, see also Yakoubi et al. 2018).

Statistical analysis

The mean value ± SD, the median with the 1st and 3rd quartile, the R², the coefficient of variation (CV), skewness and variance were given for each parameter. The p-value was considered significant only if p<0.05. Boxplots were generated to investigate inter-individual differences for each structural parameter (Fig. 11). The non-parametric Kruskal-Wallis H-test analysis was computed, using InStat (GraphPad Software Inc., San Diego, CA, USA), as some of the analyzed parameters were not normally distributed as indicated by the skewness. Correlation graphs between several structural parameters were generated.

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