Ultrastructural sublaminar-specific diversity of excitatory synaptic boutons in layer 1 of the adult human temporal lobe neocortex

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Abstract

Layer (L)1, beside receiving massive cortico-cortical, commissural and associational projections, is the termination zone of tufted dendrites of pyramidal neurons and the area of Ca^{2+} spike initiation. However, its synaptic organization in humans is not known. Quantitative 3D-models of excitatory synaptic boutons (SBs) in layer 1 of the human temporal lobe neocortex were generated from non-affected neocortical biopsy tissue using transmission electron microscopy, 3D-volume reconstructions and TEM tomography. Particularly, the size of active zones (AZs) and the readily releasable, recycling and resting pool of synaptic vesicles (SVs) were quantified.

The majority of excitatory SBs contained numerous mitochondria comprising ~7% of the total volume, had a large macular, non-perforated AZ (~0.20 μm^2) and were predominantly located on dendritic spines. Excitatory SBs had a total pool of ~3500 SVs, a comparably large readily releasable (~4 SVs), recycling (~470 SVs) and resting (~2900 SVs) pool. Astrocytic coverage of excitatory SBs suggests both synaptic cross talk or removal of spilled glutamate by astrocytic processes at synaptic complexes.

The structural composition of SBs in L1 may underlie the function of L1 networks that mediate, integrate and synchronize contextual and cross-modal information, enabling flexible and state-dependent processing of feedforward sensory inputs from other layers of the cortical column.

Key words: human neocortex, layer 1, synaptic complexes, quantitative 3D-models of excitatory synaptic boutons, astrocytic coverage, electron microscopy, TEM tomography.

Introduction

The mammalian neocortex is the most complex part of the brain comprising over 75% of the gray matter. One fundamental feature established during development is the formation of distinct layers with different networks of neurons, and its organization into vertically oriented slabs, so-called cortical columns (Marin-Padilla 1978, 1998; reviewed by Rockland and DeFelipe 2018). This organization is established in an 'inside layers first-outside layers last' fashion (Luskin and Shatz 1985a, b; reviewed by Cooper et al. 2008). The exception, beside layer (L)6 is L1 generated first originating from the marginal zone (Marin Padilla 1998; reviewed by Bystron et al. 2008). Ongoing cortico- and synaptogenesis finally lead to the formation of the adult cortical network with its unique structural and functional properties (reviewed by Lübke and Feldmeyer 2007; Rockland and DeFelipe 2018).

Remarkably, L1 is highly conserved across cortical areas and higher mammalian species, but its importance was long underestimated. However, L1 is now considered as the predominant input layer for top-down information, relayed by a rich, dense network of cortico-cortical, cortico-thalamic, commissural and associational long-range axonal projections. In humans, and thus different to experimental animals, both L1 excitatory Cajal-Retzius (CR) cells (Anstötz et al. 2014) and a quite heterogeneous population of inhibitory neurons (see for example Jiang et al. 2013; Boldog et al. 2018; Obermayer et al. 2018; Kwon et al. 2019; reviewed by Huang et al. 2024) provide signals to the terminal tuft dendrites of pyramidal neurons of the underlying cortical layers. Thus, L1 is a central locus of neocortical associations controlled by distinct types of inhibition and feedforward excitation (Schumann et al. 2019; Hartung and Letzkus 2021, reviewed by Schumann et al. 2021).

The temporal lobe neocortex (TLN) is situated on the basolateral aspect of the cerebral hemispheres primarily observed in higher primates, including humans. Notably, in humans, the TLN encompasses approximately 20% of the entire cerebral cortex (Kiernan 2012). It is recognized as a highly specialized associative neocortex, characterized by its homotypic granular six-layered organization (von Economo and Koskinas 1925; Palomero-Gallagher and Zilles 2019). The human TLN plays a crucial role in various cognitive functions, including auditory, visual, vestibular, linguistic, and olfactory processing.

Additionally, it is intricately connected to diverse sensory and multimodal associational brain regions, such as the limbic system, amygdala, and various subcortical structures (Insausti 2013). Lastly, the human TLN serves as the epicenter and onset site for temporal lobe epilepsy (Allone et al. 2017; Tai et al. 2018).

One of the most important discoveries in neuroscience, beside the definition of the neuron by Cajal's neuronal doctrine (1911), was the introduction of the term 'Synapse', more than 100 years ago. Since then these structures have been investigated from different viewpoints including structural, functional, molecular and computational studies summarized in meanwhile thousands of original publications, reviews and numerous textbooks. Yet, the quantitative geometry of the most prevalent synapse in the brain, the cortical synapse, has remained largely unexplored structurally, in both experimental animals (but see Rollenhagen et al. 2015, 2018; Bopp et al. 2017; Hsu et al. 2017; Prume et al. 2020) and, even more so, in the context of the human brain (but see Yakoubi et al. 2019a, b; Dominguez-Alvaro et al. 2019, 2021; Cano-Astorga et al. 2021, 2023; Schmuhl-Giesen et al. 2022; Shapson-Coe et al. 2024). This could be partially attributed to the availability of human brain tissue samples. Nevertheless, detailed and quantitative descriptions of synaptic complexes in L1 of the human brain are to date non-existing.

Here, an investigation into the synaptic organization of L1 in the human TLN was undertaken using high-resolution transmission electron microscopy (TEM), 3D-volume reconstruction, to generate quantitative 3D-models of synaptic boutons (SBs), and TEM tomography. The ultimate objective of this comprehensive project is to elucidate the synaptic organization, layer by layer, of the cortical column in humans.

Such meticulous quantitative structural analyses of SBs and their corresponding target structures are indispensable for comprehending, elucidating, and establishing connections between the structural and functional properties of the diverse signal cascades underlying synaptic transmission, efficacy, strength, and plasticity. Consequently, these efforts contribute significantly to the understanding of the computational properties of brain networks, in particular the synaptic organization of the cortical column.

Results

Neural and synaptic composition of L1 in the human TLN

L1 in the adult mammalian neocortex represents a relatively cell sparse layer, that in humans can be subdivided into two sublaminae, L1a and L1b, as revealed in semithin sections (Figure 1A) and Golgi-preparations (Figure 1B, C, F, G), as well as at the EM level (Figure 1H, I). L1a is more dominated by a dense network of astrocytes of different shape and size, their fine processes (Figure 2A), the occurrence of reactive microglia (Figures 1H, I, 2A) and terminal tuft dendrites of pyramidal neurons throughout the neuropil of L1 (Figures 1H, I, 2B).

Hence, L1a can be described as a sublamina with a predominant proportion of astrocytes, microglia and their processes, although it also harbors numerous other structural elements such as terminal tuft dendrites and synaptic complexes. These complexes consist of a SB juxtaposed with either a dendritic shaft or spine (Figure 2A) directly beneath or interwoven among the fine astrocytic processes within L1 (Figure 2A). Lipofuscin granules, indicative of aging, were frequently noted in astrocytes, reactive microglia (Figure 2A) and L1 neurons, although their abundance, size, and morphology varied considerably.

Remarkably, and in contrast to rodents, a persistent subpopulation of excitatory CR-cells was found in humans, identifiable by the horizontal bipolar orientation with intralaminar long-range horizontal axonal collaterals with a transcolumnar projection (Figure 1E-H; Retzius 1893, 1894; Anstötz et al. 2014; reviewed by Martinez-Cerdeno and Noctor 2014). The majority were located directly underneath the pial surface (Figure 1F, G) or intermingled with a heterogeneous population of GABAergic interneurons (Figure 1E).

In the human TLN, GABAergic interneurons constitute the predominant class of neurons in L1, exhibiting significant structural and functional heterogeneity (Figure 1D, E; see also Jiang et al, 2013, Verhoog et al. 2016; Boldog et al. 2018; Obermayer et al. 2018; Kwon et al. 2019; Schuman et al. 2019, reviewed by Huang et al. 2024) which were found throughout both sublaminae and were sometimes organized in clusters of 3-6 neurons (Figure 1D). Beside CR-cells and GABAergic interneurons, also degenerating neurons, identifiable by their shrunken and dark appearance (Figure 1A, I) were found in both sublaminae although their numbers varied substantially between brain tissue samples.

130 L1b is primarily dominated by dendritic and synaptic profiles due to a massive increase in synaptic
131 complexes (Figures 1H, 2B). Furthermore, L1b is infiltrated by apical dendrites of L2, L3 and L5 pyramidal
132 neurons interspersed with smaller dendritic segments representing apical oblique dendrites (Figures 1I, 2B).
133 The majority of synaptic complexes in L1 were axo-spinous (~80%, Figure 2 B, D, E), the remainder were
134 axo-dendritic, some of which but not all were regarded as putative GABAergic terminals (Figure 2C; but
135 see Silver et al. 2003).

136 L1b can be separated from L2 by the sudden increase of neuronal cell bodies and thick apical dendrites
137 originating at the upper pole of the somata of pyramidal neurons (Figures 1A, 2B). At the L1b and L2
138 transition more and more apical and apical oblique dendrites of different caliber were observable.

139

140 **Synaptic organization of L1 in the human TLN**

141 The primary objective of this study was to investigate the synaptic organization of excitatory SBs in L1 in
142 the human TLN, focusing on synaptic parameters that represent possible morphological correlates of
143 synaptic transmission, efficacy, strength and plasticity. The quantitative analysis was separated for both
144 sublaminae to look for possible sublaminar-specific differences. Beside the overall geometry of SBs and the
145 number and size of mitochondria, in particular the size of the PreAZ and PSD constituting the AZ, the
146 morphological equivalent to a functional neurotransmitter release site, and the organization of the three
147 functionally defined pools of SVs, namely the readily releasable (RRP), the recycling (RP) and resting pool,
148 were quantified.

149 To achieve this, a total of 361 SBs was completely reconstructed out of four patients in six series
150 containing 100-150 ultrathin sections/series. A total of 190 SBs and 184 AZs (L1a) and 171 SBs and 165
151 AZs (L1b) using non-affected neocortical access tissue (see Material and Methods; Source Data 02) were
152 completely 3D-reconstructed and quantified using fine-scale TEM, digital TEM images and subsequent 3D-
153 volume reconstructions.

154 Excitatory and inhibitory synaptic complexes in L1a und L1b were formed by either presynaptic *en*
155 *passant* or endterminal boutons with their prospective postsynaptic target structures dendritic shafts (Figures

156 2B C, D, 3A-C) or spines of different caliber and type (Figures 2B, D, E, 3A-C). In both sublaminae, SBs
157 were predominantly found on dendritic spines (~81%; L1a) and ~79% (L1b), the remainder were located
158 on dendritic shafts. The majority of SBs on spines were located on mushroom spines (L1a: ~83%, L1b:
159 ~73%), a smaller fraction on stubby spines (L1a: ~11%, L1b: ~9%) or on thin elongated spines (L1a: 6%,
160 L1b: ~22%; Figure 2E), the remainder were not classifiable.

161 Since our focus was on excitatory SBs, inhibitory terminals were not further analyzed in detail. Inhibitory
162 SBs were distinguished from excitatory SBs by their smaller SVs, which have a more spherical appearance,
163 a smaller sized PreAZ, and the lack of a prominent PSD (Figure 2D). Infrequently, GABAergic and
164 glutamatergic terminals were found on the same dendrite (Figure 2C, D) or spine (see for example Wittner
165 et al. 2001) but less frequently at ~2-3%. (Kwon et al. 2019). In turn, not all SBs located on dendritic shafts
166 were GABAergic (see for example Silver et al. 2003).

167 The ratio between excitatory vs. inhibitory SBs was between 10-15% although with layer-specific
168 differences. Our findings are in good agreement with recent publications by Shapson-Coe et al. (2024) and
169 by Cano-Astorga et al. (2023, 2024) in which the number of inhibitory SBs in different layers of the temporal
170 lobe neocortex was estimated to be10-15%. No significant differences were found in the content of
171 inhibitory SBs between the two sublaminae L1a and L1b.

172 Some SBs in both sublaminae were seen to establish either two or three synapses on the same spine,
173 spines of other origin, or dendritic shafts. Remarkably, ~90% of spines in L1a and L1b contained a spine
174 apparatus (Figure 2E), a specialized form of the endoplasmic reticulum, structures that increase spine
175 motility and may also stabilize the synaptic complex during signal transduction (Deller et al. 2003; reviewed
176 by Knott and Holtmaat 2008).

177 Remarkably, beside synaptic complexes, also 'special connections' in L1 of the human TLN were
178 observed. This encompassed so-called dendro-dendritic synapses and fine astrocytic processes that receive
179 synaptic input from SBs and in turn, provide synaptic output to either dendritic shafts or spines (not shown,
180 but see Yakoubi et al. 2019a, b; Schmuhl-Giesen et al. 2022).

181

182 **Synaptic density in L1a and L1b of the human TLN**

183 Synaptic density measurements serve as a valuable tool for quantitatively characterizing the synaptic
184 organization of a specific cortical layer, assessing the connectivity rate, and identifying potential inter-
185 individual differences among patients within the human TLN.

186 The average density of synaptic complexes in L1 was comparatively high with a value of $5.26*10^8 \pm$
187 $8.44*10^7$, $5.52*10^8 \pm 1.25*10^8$ synapses/mm^3 in L1a and $5.01*10^8 \pm 1.40*10^7$ synapses/mm^3 in L1b, ranging
188 from $4.08*10^8$ to $6.31*10^8$ (L1a) and from $4.87*10^8$ to $5.15*10^8$ (L1b), respectively (Supplemental Table
189 1; Source Data 01). Strikingly, a huge inter-individual variability was found, although with no significant
190 difference between L1a and L1b.

191 Therefore, L1 exhibited a comparably high density of synaptic complexes, indicating a relatively robust
192 and thus strong connectivity of neurons within L1 of the human TLN.

193

194 **Geometry and size of SBs and mitochondria in the human TLN**

195 Overall, L1 SBs were on average medium-sized, with a mean surface area of 5.48 ± 1.40 µm^2, and a mean
196 volume of 0.50 ± 0.19 µm^3, with a slight difference in size between L1a and L1b. Interestingly, the
197 variability in both surface area and volume of SBs was comparably small in L1a and L1b as indicated by a
198 low CV and variance (Table 1; Source Data 02) regardless of their target structures. SBs in L1 were
199 comparable in size with SBs in L5, L6, but ~2-fold larger than those in L4 of the human TLN ($p \leq 0.001$;
200 see also Yakoubi et al. 2019a, b: Schmuhl-Giesen et al. 2022).

201 Mitochondria play a pivotal role in synaptic transmission and plasticity (reviewed by Dallerac et al. 2018;
202 see also Discussion). In L1 SBs either no (Figures 2B, 3A, E) or several mitochondria (Figures 2, 3C, D;
203 range 0 to 8) of different shape and size were observed. When present mitochondria had a volume of 0.04
204 $\pm$ 0.02 µm^3 in L1a and 0.05 ± 0.01 µm^3 in L1b, respectively (Table 1; Source Data 02). Mitochondria
205 contributed with ~7% (L1) to the total bouton volume of SBs, similar to values in L6 (~6%; Schmuhl-Giesen
206 et al. 2022), but with a ~1.5-fold lower percentage as those in L5 (~12%; Yakoubi et al. 2019a) and L4
207 (~13%; Yakoubi et al. 2019b).

208 A good correlation between the volume of the SBs and that of mitochondria was found for L1a (R^2: 0.7528;
209 Figure 4B) and for L1b (R^2: 0.5992; Figure 4B). A weak correlation was also found for L6 (Schmuhl-Giesen
210 et al. 2022) but a strong one in both L4 (Yakoubi et al. 2019a) and L5 (Yakoubi et al. 2019b) suggesting a
211 layer-specific difference in the content of mitochondria in individual SBs.
212
213 **Structural composition of AZs in L1a and L1b excitatory SBs in the human TLN**
214 The number, size, and shape of the AZ, composed of the PreAZ and PSD, is one key structural determinant
215 in synaptic transmission and plasticity (Südhof 2002; Matz et al. 2010; Holderith et al. 2012) as it represents
216 the docking area of SVs (PreAZs) and the receiving area of neurotransmitter quanta at the PSD (Südhof
217 2012).
218 The majority (~98%) of SBs in L1a and L1b had only a single (Figures 2C-E, 3A-C, E) at most two or
219 three AZs (Figure 3D). Beside very large, spanning the entire pre- and postsynaptic apposition zone, also
220 smaller AZs covering only a fraction of the pre- and postsynaptic apposition zone were found.
221 On average, PreAZs were 0.18 ± 0.08 μm^2 in surface area in L1a and 0.22 ± 0.04 μm^2 in L1b with only
222 a slight variability between both sublaminae. The surface area of PSDs was quite similar to that of PreAZs
223 0.21 ± 0.01 μm^2 and was in L1a and 0.22 ± 0.05 μm^2 in L1b, respectively (Table 1; Source Data 02, sheet
224 01). Thus, the similar size of the PreAZ and PSD showed a nearly perfect overlap at the pre- and postsynaptic
225 apposition zone. L1 AZs did not show a large variability in size as indicated by the low SD, CV, and variance
226 (Table 1). Remarkably, AZs exhibited a wide range of shapes in addition to different sizes. Two types of
227 AZs were found: The majority in L1 (~80%) were of the macular and non-perforated type (Figure 3D), the
228 remainder being either horseshoe-shaped or ring-like and perforated (Figure 3E).
229 Notably, our analysis revealed only a weak correlation between the surface area of SBs and that of
230 PreAZs for L1a and L1b SBs (Figure 4A). These findings imply that the size of SBs and PreAZs is regulated
231 independently .
232 The size of the synaptic cleft (Table 1; Source Data 02, sheet 03) is an important measure at AZs since
233 its size (diameter) critically determines the temporal and spatial neurotransmitter concentration.

234 Measurements of the size of the synaptic cleft were performed on random EM images taken from the series
235 using OpenCAR or online directly at the TEM using ImageSP (Fa. Tröndle, Moorenweis, Germany). Only
236 synaptic clefts cut perpendicular to the PreAZ and PSD were included in the sample. The distance between
237 the outer edge of the PreAZ and PSD was measured at the two lateral edges and separately at the central
238 region of the synaptic cleft; the two values of the lateral edges were averaged for each cleft measurement.
239 Finally, a mean ± SD was calculated for both the lateral and central region over all synaptic clefts analyzed
240 per patient.

241 The size of the synaptic cleft did not differ significantly between the two sublaminae and was similar to
242 the results in L4, L5 (Yakoubi et al. 2019a, b) and L6.Schmuhl-Giesen et al. 2022).

243

244 **Organization of the pools of SVs in L1a and L1b excitatory SBs of the human TLN**

245 SVs are the other key structure in neurotransmitter storage and release, hence they play a fundamental role
246 in synaptic transmission and in the modulation of short- and long-term synaptic plasticity (Südhof 2002,
247 2012). Three different pools of SVs are functionally defined: the RRP, the RP, and the resting pool. Synaptic
248 efficacy, strength, mode, and probability of release (P_r) are regulated by these pools (Schikorski and Stevens
249 2001; Silver et al. 2003; Rizzoli and Betz 2004; Saviane and Silver 2006; Schikorski 2014; Watanabe et al.
250 2014; Neher 2015; Vaden et al. 2019; reviewed by Rizzoli and Betz 2005; Denker and Rizzoli 2010;
251 Chamberland and Toth 2016).

252 In general, SVs were distributed throughout the entire terminal in ~95% of the population of SBs
253 investigated (Figure 2, 3), the remainder showed a more cluster-like distribution. Two different types of
254 vesicles were found: (1) Small clear SVs with an average diameter of 25.03 ± 5.27 nm ranging up to ~40
255 nm (Table 1; Source Data 02, sheet 01). Using TEM tomography, the SV diameter was on average 30.29 ±
256 4.67 nm ranging up to ~47 nm (n = 38 SBs with a total of 5.834 SVs; Table 1; Source Data 03, sheet 05).
257 (2) DCVs (Dense Core Vesicles; Figure 3B, C, E; Source Data 02, sheet 01) with an average diameter of
258 57.97 ± 6.45 nm with no significant difference between both sublaminae. DCVs were intermingled with the
259 population of SVs throughout the entire SB (Figure 3B, C, E). DCVs play an important role in endo- and

260 exocytosis by releasing various co-transmitters, such as neuropeptides, ATP, noradrenalin, and dynorphin
261 (Ghijsen and Leenders 2005) or in synaptogenesis (Sorra et al. 2006) and the build-up of PreAZs by
262 releasing Piccolo and Bassoon (Zhai et al. 2001; Shapira et al. 2003) In addition, they are involved in
263 clustering SVs at the PreAZs (Mukherjee et al. 2010; Watanabe et al. 2014).

264 The distribution pattern of SVs made it impossible to morphologically distinguish between the three
265 functionally defined pools of SVs, with the exception of the so-called 'docked' ones, that are fused to the
266 PreAZ, and the omega-shaped bodies, that have already opened and released quanta of neurotransmitter
267 (Rizzoli and Betz, 2004; Denker and Rizzoli 2010; Neher 2015; reviewed by Rizzoli and Betz 2005;
268 Chamberland and Toth 2015). However, an attempt was made to define a morphological correlate for the
269 three functionally defined pools.

270 For this purpose, a perimeter analysis (a modified Sholl-analysis) of each SV in individual SBs was
271 carried out. The perimeter was defined as the minimum distance between each SV membrane and the PreAZ
272 membrane (Sätzler et al. 2002; for criteria to define SV pools see also Rizzoli and Betz 2005; Denker and
273 Rizzoli 2010). The putative RRP was defined as a perimeter (p) p≤10 nm and p≤20 nm to the PreAZ. These
274 criteria for the RRP from which SVs could be easily and rapidly recruited were chosen because both values
275 are less than a SV diameter apart from the PreAZ. The putative RP maintains release during moderate
276 stimulation and was classified at a distance from the PreAZ between p60-p200 nm. All SVs that were further
277 than 200 nm from the PreAZ were regarded to constitute the putative resting pool which acts as a deposit
278 of SVs only used by intense and/or repetitive stimulation.

279 On average, the total pool of SVs was 2958.62 ± 1940.51 in L1a vs. 3903.32 ± 1852.23 in L1b with an
280 average of 3430.97 ± 1773.77 for L1 (Table 1; Source Data 02). Remarkably, the total pool in L1b was
281 significantly larger ($p \leq 0.01$) by ~1.3-fold when compared to L1a. In both sublaminae (Figure 3D, E), there
282 is a huge variability in total pool size , ranging from 758 to 6542 in L1a and 1507 to 15060 in L1b, as
283 indicated by the SD, IQR and variance (Table 1). SVs contributed with ~5% (0.03 μm^3) in L1a and ~4%
284 (0.02 μm^3) in L1b to the total volume of SBs. However, no significant difference between both sublaminae
285 was observed (Table 1; Source Data 02).

286 Interestingly, no correlation between the volume of SBs vs. the total pool size was found for L1a (R^2:
287 0.2490), but a good correlation for L1b (R^2: 0.5700). Furthermore, no correlation was found between the
288 PreAZ vs. the total pool size (Figure 4C; Source Data 02).

289 The average number of SVs in the putative RRP/PreAZ for L1was 3.60 ± 4.24 SVs at p10 nm (L1a: 5.90
290 ± 5.05 SVs and L1b: 1.29 ± 1.87 SVs) with a significant difference ($p \leq 0.01$) by ~4.5-fold between the two
291 sublaminae (Table 1; Source Data 02). The putative RRP/PreAZ at the p20 nm criterion was on average
292 19.05 ±17.23 SVs (L1a: 25.04 ± 21.09 SVs and L1b: 13.07 ± 13.87 SVs) and thus nearly 2-fold larger for
293 L1a, although with a huge variability, but even though may point to sublaminae-specific differences in P_r,
294 synaptic efficacy, strength and paired-pulse behavior at individual SBs.

295 No correlation between the putative RRP at both the p10 nm and p20 nm criterion and the surface area
296 of PreAZs was found for both sublaminae. (Figure 4D, E).

297 The putative RP/PreAZ at the 60-200 nm perimeter criterion was 390.12 ± 286.89 SVs in L1a and 535.88
298 ± 321.16 in L1b with no significant difference for both sublaminae (Table 1; Source Data 02, sheet 01). It
299 should be noted however, that the variability of the putative RP is relatively large in both sublaminae as
300 indicated by the SD, median, IQR and variance (Table 1).

301 Again, no correlation was observed between the SVs in the putative p60-200 nm RP and the surface area
302 of the PreAZs (L1a, Figure 4F) and for L1b (Figure 4F; Source Data 02, sheet 01), respectively.

303 The putative resting pool of SVs contained on average 2515.02 ± 1588.99 SVs in L1a and 3277.97 ±
304 1480.96 SVs in L1b with a significant difference between the two sublaminae ($p \leq 0.01$, Table 1; Source
305 Data 02, sheet 01). The number of SVs in the putative resting pool in L1b was about 1.3-fold larger when
306 compared to L1a. Again, also the putative resting pool showed a large variability for both sublaminae as
307 indicated by the SD, CV, IQR and variance (Table 1).

308 No correlation was observed between the SVs in the putative p200 nm and p500 nm resting pool and the
309 surface area of the PreAZs in L1a and L1b (Figure 4G, H; Source Data 02, sheet 01).

310 Neither for the total nor for the three different putative pools of synaptic vesicles significant differences
311 between SBs terminating on dendritic spines or shafts were found (Source Data 02, sheet 04).

312 **TEM tomography of L1 excitatory SBs in the human TLN**

313 It is still controversially discussed whether so-called 'docked' SVs or omega-shaped bodies represent the
314 RRP. To compare our results from the perimeter analysis for the p10 nm RRP with that of 'docked' SVs,
315 high-resolution TEM tomography was carried out. Only SBs where the AZ could be followed from its
316 beginning to its end in individual tilt-series and where the AZ was cut perpendicular through the PreAZ,
317 PSD and the synaptic cleft were analyzed. In L1a (50 SBs, on dendritic shafts = 25 SBs; on spines = 25
318 SBs) and L1b (50 SBs, on dendritic shafts = 25 SBs; on spines = 25 SBs) the number of 'docked' SVs was
319 analyzed at the PreAZ (Figure 5; Table 2; Source Data 03).

320 The results for L1a and L1b were two-fold: First, in agreement with already published results (Yakoubi
321 et al. 2019a, b; Schmuhl-Giesen et al. 2022) only in a minority (<1%) of all PreAZs analyzed, regardless of
322 their target structures, a dendritic shaft (Figure 5A) or spine (Figure 5B, C), no 'docked' SVs were observed,
323 which is in contrast to our perimeter analyses showing several boutons without vesicles within the p10 nm
324 criterion. Second, the majority of PreAZs (~98%) contained more than 2, the most 8 (L1a) and 6 (L1b)
325 'docked' SVs. This finding strongly supports multivesicular release of 'docked' SVs in L1a and L1b SBs
326 in line with findings in L4, L5 and L6 SBs (Yakoubi et al. 2019a, b; Schmuhl-Giesen et al. 2022; see also
327 Figure 5D-F).

328 On average, 3.71 ± 1.38 'docked' SVs (L1a) and 3.42 ± 1.34 'docked SVs' (L1b) were found at
329 individual PreAZs with similar values for both sublaminae. Furthermore, no significant difference was
330 found for 'docked' SVs between shaft vs. spine synapses in L1. The number of 'docked' SVs at L1a PreAZs
331 was ~2-fold smaller but nearly 3-fold larger in L1b when compared to the results of our quantitative
332 perimeter analysis for the putative RRP using the p10 nm criterion. In contrast to the p10 nm criterion with
333 high inter- and intra-individual variabilities (Suppl. Fig. 2), the number of 'docked' SVs showed a relatively
334 low variability as indicated by the SD, CV, variance, and skewness. They were different ($p \leq 0.001$) from
335 the values estimated with the p10 nm perimeter analysis.

336 There seems to be a tendency that larger PreAZs contained more 'docked' SVs providing a larger
337 'docking' area allowing the recruitment of more SVs.

338 In summary, a notable disparity difference was observed between values obtained for L1 using the p10 nm
339 criterion of the perimeter- and the TEM tomography analysis. Additionally, the putative RRP in L1 (this
340 study) and L6 (Schmuhl-Giesen et al. 2022) was ∼2-4-times smaller when compared to values in L4
341 (Yakoubi et al. 2019b) and L5 (Yakoubi et al. 2019a) pointing towards a layer-specific regulation of the
342 putative RRP. Moreover, the results suggest that not only 'docked' SVs but also those very close to the
343 PreAZ should be considered to belong to the putative RRP.
344
345 **Astrocytic coverage of L1 SBs in the human TLN**
346 Astrocytes, by directly interacting with synaptic complexes thus forming the 'tripartite' synapse play a
347 pivotal role in the induction, maintenance and termination of synaptic transmission by controlling the spatial
348 and temporal concentration of neurotransmitter quanta in the synaptic cleft (reviewed by Dellerac et al.
349 2018). Astrocytic profiles were identified by their irregular stellate shape, relatively clear cytoplasm,
350 numerous glycogen granules and bundles of intermediate filaments (for criteria see for example Peters et al.
351 1991; Ventura and Harris 1999; see also Figure 6A, B) Astrocytes and their fine processes formed a
352 relatively dense (in L4, L5), but a comparably loose network within the neuropil in L1 (Figure 6A-C; Source
353 Data 04, sheet 01) by contributing with ~20% to the total human TLN volume, with the highest degree in
354 coverage in L4 but similar to findings in L6 of the human TLN (Figure 6C; Source Data 04, sheet 01).
355 In general, the majority (~70% both in spine and shaft synapses; Figure 6D, Source Data 04, sheet 02)
356 of synaptic complexes in L1 were without astrocytic coverage close to the AZ or only partially ensheated
357 (~20% both in spine and shaft synapses) by fine astrocytic processes. In only a small fraction (~10%) of
358 both spine and shaft synapses fine astrocytic processes were observed to form the 'tripartite' synapse that
359 physically isolate synaptic complexes from the surrounding neuropil. The findings in L1 (L1a: n=3 patients,
360 n=128 spine synapses, n= 48 shaft synapse; L1b: n=3 patients, n=126 spine synapses, n= 44 shaft synapses)
361 are in contrast to L4 (n=5 patients; n=93 spine synapses, n= 41 shaft synapses) were a higher number of
362 'tripartite' synaptic complexes (spines: ~50%; shafts: ~20%) was found. In L4, ~ 25% of AZs on dendritic
363 spines and ~ 60% on dendritic shafts were partially covered, with a huge inter-individual difference. The

364 remainder were not covered. No significant differences were found between spine and shaft synapses in L1
365 or L4 (Figure 6D, Source Data 04, sheet 02).
366 In summary, it is most likely that the partial coverage or complete absence of fine astrocytic processes
367 at the majority of synaptic complexes in L1 of the human TLN may contribute to different 'behaviors' of
368 synaptic complexes in synaptic transmission and in the modulation of short-term plasticity, for example in
369 the removal of 'spilled' horizontally diffusing neurotransmitter quanta favoring synaptic crosstalk (for more
370 detail see Discussion).
371
372
373 **Discussion**
374 This study investigated the synaptic organization of L1 in the human TLN. The quantitative 3D-models
375 analyzed showed beside similarities significant layer-specific differences in structural and synaptic
376 parameters, in particular in the size of the AZs and the total pool of SVs, the putative RRP, RP and resting
377 pool when compared with other already investigated layers (Yakoubi et al. 2019a, b; Schmuhl-Giesen et al.
378 2022). The low degree of astrocytic coverage of L1 SBs suggests that glutamate spillover and as a
379 consequence synaptic cross talk may occur at the majority of synaptic complexes in L1
380
381 **Synaptic density measurements**
382 Synaptic density measurements are a useful tool to describe the synaptic organization of a particular area,
383 nuclei and even layers in different brain regions, but also the degree of connectivity underlying the
384 computational properties of a given brain area or in a given brain network. Meanwhile numerous studies in
385 various animal species and brain regions have performed such an analysis but data for a density of synaptic
386 complexes in humans are still rare (but see Marco and DeFelipe 1997; Tang et al. 2001; DeFelipe et al.
387 1999, 2002; Alonso-Nanclares et al. 2008; Blazquez-Llorca et al. 2013; Finnema et al. 2016; Cano-Astorga
388 et al. 2021, 2023).

389 Strikingly, a huge layer-specific difference in the mean density of synaptic contacts was found in the human
390 TLN (see also Supplemental Table 1). In L1, the overall density was $5.26*10^8 \pm 8.44*10^7$ synaptic
391 complexes/mm^3 although a great variability was observed as indicated by the SD consistent with findings
392 in the other layers of the human TLN L4 ($2.37*10^6 \pm 2.19*10^6$; Yakoubi et al. 2019b); L5 ($3.89 *10^8 \pm$
393 $9.12*10^8$; Yakoubi R., PhD Thesis 2023) and L6 ($4.98*10^7 \pm 1.85*10^7$; Schmuhl-Giesen et al. 2022) and
394 existing data published by Alonso-Nanclares et al. 2008: $9.13 \pm 0.63*10^8$ and Tang et al. 2001: $164*10^{12}$.
395 The density values for L1 and L5 are in the same order of magnitude but differ substantially from L4 and
396 L6 by one or two orders of magnitude. The huge differences in synaptic density may be explained by gender
397 and age-specific differences and the use of different methods in estimating synaptic densities (DeFelipe et
398 al. 1999).

399 In summary, the highest synaptic density was observed in L1, indicating a comparably high level of
400 connectivity and synaptic interaction. This may suggests that L1 facilitates rapid information processing
401 due to its robust synaptic activity.

402

403 **Important structural subelements of SBs in the human TLN**

404 **Shape and size of PreAZs and PSDs**

405 Synaptic efficacy, strength, modes of release and P_r are beside the pool of SVs, determined by the shape
406 and size of AZs (Matz et al. 2010; Holderith et al. 2012; Südhof 2012).

407 The majority of SBs in L1 of the human TLN had a single at most three AZs that could be of the non-
408 perforated macular or perforated type. These findings are comparable with results for other layers in the
409 human TLN (L4: Yakoubi et al. 2019b, L5: Yakoubi et al. 2019a, L6: Schmuhl-Giesen et al. 2022) but by
410 ~1.5-fold larger than in rodent and non-human primate neocortex (Marrone et al. 2005; Rollenhagen et al.
411 2015, 2018; Bopp et al. 2017; Hsu et al. 2017).

412 The surface area of AZs in L1 SBs of the human TLN was on average ~0.20 μm^2. This is in good
413 agreement with data obtained for AZs in L3 (Cano-Astorga et al. 2021), L4 (Yakoubi et al. 2019b), L5

414 (Yakoubi et al. 2019a), L6 (Schmuhl-Giesen et al. 2022) and in L3 (Cano-Astorga et al. 2023) of the human
415 temporal and cingulate neocortex. However, they were ~2- to 3-fold larger than those in mouse and non-
416 human primates visual, motor, and somatosensory neocortex (Bopp et al. 2017; Hsu et al. 2017) but
417 remarkably even larger than AZs in comparably large CNS terminals like the hippocampal mossy fiber
418 bouton (Rollenhagen et al. 2007) the cerebellar mossy fiber bouton (Xu-Friedman and Regehr 2003)and the
419 Calyx of Held in the medial nucleus of the trapezoid body (Sätzler et al. 2002).

420 The substantial variability in the size of AZs at individual SBs, observed both in the human TLN and in
421 experimental animals, likely contributes to differences in synaptic efficacy, strength, P_r, quantal size, as
422 well as the size of the RRP and RP (Südhof 2002; Matz et al. 2010, Freche et al. 2011; Holderith et al. 2012;
423 Neher 2015; Chamerland and Toth 2016; Rollenhagen et al. 2018; Vaden et al. 2019; reviewed by Rizzoli
424 and Betz 2005; Denker and Rizzoli 2010). However, it has to be noted that the size of the AZ is regulated
425 as a function of activity, as shown for hippocampal SBs in the CA1 subregion (Matz et al. 2010; Holderith
426 et al. 2012). However, the comparably large size of the AZs in the human TLN also suggest a larger
427 'docking' area allowing the fusion of more SVs and thus a larger P_r.

428 It has be postulated that perforated AZs contribute to synaptic efficacy (Geinismann et al. 1992; Nava et
429 al. 2014) by an increased number of SVs at perforated synapses and plasticity (Buchs and Muller 1996;
430 Desmond and Weinberg; 1998) and by increasing the number of AMPA- and NMDA-type glutamate
431 receptors at perforated synapses (Ganeshina et al. 2004). However, since the number of perforated AZs in
432 all layers of the human TLN is comparably low compared to those of the macular-non-perforated type, it is
433 highly speculative which impact they might have here on synaptic efficacy and the modulation of synaptic
434 plasticity.

435

436 **Size of SVs obtained with TEM vs. TEM tomography**

437 The values obtained with both TEM (25.03 ± 5.27 nm) and TEM tomography (30.29 ± 4.67 nm) in L1 are
438 within a range published in previous studies (e.g. human TLN: Yakoubi et al. 2019a, b; Schmuhl-Giesen et
439 al. 2022; rat hippocampal CA3 region: Rollenhagen et al. 2007; Zhao et al. 2012a; rodent hippocampal CA1

440 region: Harris and Sultan 1995 [lower range]; rodent somatosensory cortex: Rollenhagen et al. 2015; 2018;
441 Prume et al. 2020). Based on TEM studies also higher mean SV diameters of ~40 nm and beyond have been
442 published for different species and brain regions (e.g. Schikorski and Stevens 1997; Sätzler et al. 2002;
443 Deak et al. 2003; Hu et al. 2008). TEM tomographic analyses also indicate variability in SV diameter from
444 ~30 to ~52 nm in different brain regions and mouse knock-out model systems (Imig et al. 2014; Muth et al.
445 2024). High variability of SV diameter have been reported to be a general phenomenon in several species
446 and brain regions, even a synapse-to-synapse variability in mean SV size is described in neighboring
447 synapses in the hippocampus, neocortex, and cerebellum of rats, mice, and human cell culture systems (Hu
448 et. al. 2008; Qu et al. 2009). Additionally, within a single SB the size of individual SVs can vary for example
449 depending on their transmitter filling state and content (e.g. Colliver et al. 2000; Pothos et al. 2002).
450

451 **Size of the three pools of SVs**

452 Besides the size of the PreAZ, the pool of rapidly releasable SVs also critically determines P_r and thus
453 synaptic efficacy, strength and plasticity (Rosenmund and Stevens 1996; Schikorski and Stevens 2001;
454 Rizzoli and Betz 2004; Schikorski 2014; Watanabe et al. 2014; Vaden et al. 2019; reviewed by Rizzoli and
455 Betz 2005, Neher 2015; Chamberland and Toth 2016). It is still rather unclear whether functionally
456 heterogeneous SV pools are structurally identifiable and thus support diverse forms of synaptic transmission
457 und would also play a pivotal role in long- and short-term plasticity. Synaptic transmission can be modulated
458 in various ways depending on the availability of SVs and on their recycling rates. Hence, the size of both
459 the RRP and RP critically determines synaptic efficacy, strength and plasticity. These parameters are
460 controlled at the PreAZ but vary substantially across various CNS synapses (reviewed by Rizzoli and Betz
461 2005; Neher 2015). The contribution of the RRP size to synaptic dynamics and the mechanisms by which
462 such control is achieved at individual SBs remains largely unknown.

463 L1-SBs had a total pool size of ~3500 SVs/AZ, larger than in other layers of the human TLN: ~2-fold in
464 L4 (~1800 SVs; Yakoubi et al. 2019b), ~2.6-fold in L5 (~1350 SVs; Yakoubi et al. 2019a) and ~3-fold in
465 L6 (~1150 SVs; Schmuhl-Giesen et al. 2022). Remarkably, the total pool sizes in L4 and L5 of the human

466 TLN were significantly larger than those in L4 (~550 SVs/AZ; Rollenhagen et al. 2015) and L5 (~750
467 SVs/AZ; Rollenhagen et al. 2018) of the rat somatosensory cortex.
468 The largest total pool size in L1 may point to a pivotal role in the rapid replenishment after depletion by
469 high-frequency stimulation and the transfer of SVs into the RRP and RP from the total pool in L1 SBs.
470 The putative RRP at L1 PreAZs was on average ~4 SVs/AZ for the p10 nm RRP and similar to that in
471 L5 (~5 SVs/AZ), but significantly smaller by ~5.3-fold to that of L4 (~20 SVs/AZ) and by ~3.5-fold when
472 compared to L6 in the human TLN. Also, huge differences were observed in the putative p20 nm RRP which
473 at L1 PreAZs was ~20 SVs, ~40 SVs in L4, ~15 SVs in L5 and ~30 SVs in L6, respectively. It has to be
474 noted that a layer-specific difference in both the putative p10 nm and p20 nm RRP exists although a huge
475 variability was observed as indicated by the SD, IQR, and variance. Hence, at L1 PreAZs the overall RRP,
476 taken the p10 nm and p20 nm criterion (both together are less than a vesicle diameter) was constituted by
477 ~25 SVs rapidly available SVs that may partially contribute to the efficacy, strength and reliability of
478 synaptic transmission, but also in the modulation of short-term synaptic plasticity in L1.
479 The size of the putative RP/PreAZ was ~470 SVs in human L1 SBs and thus ~3-fold larger to that in L6
480 SBs, ~1.3- fold larger than in L4, and ~2.4-fold larger than in L5 SBs of the human TLN. In the rodent
481 neocortex the RP/PreAZ comprised ~130 (L4; Rollenhagen et al. 2015) and ~200 SVs (L5; Rollenhagen et
482 al. 2018). The comparably largest size of the RP in L1 when compared with values for the other cortical
483 layers in the human TLN and that found for L4 and L5 in rodents may also point to a rapid availability of
484 SVs from the putative RP after the replenishment of the putative RRP during high-frequency stimulation. If
485 the refilling rates were activity dependent, the large size of the RP at L1 PreAZs could explain some forms
486 of short-term synaptic plasticity, e.g. a substantial increase in synaptic strength during frequency facilitation
487 and post-tetanic potentiation at these SBs.
488 Finally, the putative resting pool of SVs at L1 PreAZs in the human TLN is also comparably large (~3000
489 SVs) and again the largest when compared with other layers in the human TLN (L4 and L5: ~1250 SVs;
490 L6: ~900 SVs). The size of the resting pool may guarantee to rapidly replenish the RRP and RP after

491 repetitive high-frequency stimulation via active transfer of SVs with the help of mitochondria associated
492 with the pool of SVs (this study, see also Zhou and Fuster 1996; Verstreken et al. 2005; Smith et al. 2016).
493 The notable disparities in AZ and SV pool sizes among individual SBs may contribute to rapid alterations
494 in the computational properties of single neurons or networks. Consequently, these variations in AZ and SV
495 pool sizes at L1 SBs may critically influence the behavior of SBs during so-called Up-and-Down states as
496 described for other SBs of the CNS (Zhou and Fuster 1996; Sanchez-Vives and McCormick 2000; Sakata
497 and Harris 2009; Testa-Silva et al. 2014).
498
499 **Importance of presynaptic mitochondria in synaptic transmission**
500 Mitochondria in the cortical layers of the human TLN were organized in clusters associated with the pool
501 of SVs (this study, see also Yakoubi et al. 2019a, b; Schmuhl-Giesen et al. 2022). In L1, mitochondria only
502 contribute by about 7.2% (L1a) and 6.7% (L1b) to the total SB volume with similar values between L1 and
503 L6, but their percentage was ~2-fold lower than values in L4 and L5 suggesting a layer-specific distribution
504 of mitochondria in the human TLN.
505 Mitochondria play a pivotal role in the recruitment and mobilization of SVs from the RP and resting pool
506 and in the priming and docking process of SVs (Verstreken et al. 2005; Perkins et al. 2010; Smith et al.
507 2016; reviewed by Dallerac et al. 2018). In the CNS they act as the main source of internal calcium (Pozzan
508 and Rizzuto 2000; Rizzuto et al. 2000), thus they regulate and control the internal calcium concentrations
509 in CNS terminals required for the signal cascades where for example, synaptic proteins driven by Ca^{2+} like
510 synaptotagmin, synaptophysin, synaptobrevin and the SNARE-complex are involved (Südhof 2002).
511
512 **Astrocytic coverage of L1 SBS in the human TLN**
513 It is widely recognized that astrocytes play a crucial role in the formation of the 'tripartite' synapse, which
514 is a common characteristic of cortical synapses. Astrocytes serve both as a physical barrier to glutamate
515 diffusion and to mediate neurotransmitter uptake via transporters, thereby regulating the spatial and
516 temporal concentration of neurotransmitters in the synaptic cleft (Oliet et al. 2004; Min and Nevian 2012;

517 Pannasch et al. 2014; reviewed by Dallerac et al. 2018). In addition, they modulate synaptic transmission
518 by activating pre- and postsynaptic receptors (Haydon and Carmignoto 2006; Le Meur et al. 2012).
519 Moreover, it was found that the control of timing-dependent long-term depression (t-LTD) at neocortical
520 synapses is critically influenced by astrocytes by increasing Ca^{2+} signaling during the induction of t-LTD
521 (Min and Nevian 2012).

522 Remarkably, significant layer-specific differences exist in the astrocytic coverage of synaptic complexes
523 in the human TLN. Whereas in L4 and L5 ~60-80% of the total volumetric fraction was occupied by
524 astrocytic processes and most of AZs in L4 were tightly ensheathed by fine astrocytic processes, in L1 and
525 L6 the volumetric fraction of astrocytic processes was only ~20% with a loose and incomplete coverage of
526 AZs in L1 (Figure 6D). As a consequence, the lack or only incomplete astrocytic coverage of synaptic
527 complexes in L1 can only partially act as a physical barrier to neurotransmitter diffusion. In addition, beside
528 a vertical also its horizontal diffusion of glutamate at the synaptic cleft is possible. Hence, two possible
529 scenarios for the role of astrocytes are present at L1 synaptic complexes. First, as shown for only a small
530 part (10%) of synaptic complexes in L1 the 'tripartite' synapse is realized at both sides of the synaptic cleft
531 (Figure 6D). This would allow the selective uptake of horizontally diffusing glutamate via glutamate
532 transporters located in the fine astrocytic processes. This prevents 'glutamate spillover', and thus allow a
533 vertically directed neurotransmitter diffusion and docking to postsynaptic receptors at the PSD.

534 The remaining larger part of synaptic complexes in L1 either lack a coverage by fine astrocytic processes
535 at AZs or are only partially ensheathed (Figure 6D). Here 'glutamate spillover' of horizontally diffusing
536 neurotransmitter quanta is most likely and thus synaptic crosstalk between neighboring synaptic complexes
537 at AZs in neighboring spines on terminal tuft dendrites because spine density in L1 is high. This may cause
538 a switch from asynchronous to synchronous release from neighboring synaptic complexes upon repetitive
539 low- and high-frequency stimulation (von Gersdorff and Borst 2002).

540 In summary, two distinct structural scenarios were identified regarding the role of astrocytes in L1,
541 However, it is still rather unknown which of the two scenarios contribute more efficiently to synaptic
542 transmission and plasticity in L1.

543 **The role of L1 in the information processing of the cortical column**

544 The computational properties of the neocortex depend upon its ability to integrate the information provided
545 by the sensory organs (bottom-up information) with internally generated signals such as expectations or
546 attentional signals (top-down information). This integration occurs at apical tuft dendrites of L2/3 and L5
547 pyramidal neurons in L1. Importantly, L1 is the predominant input layer for top-down information, relayed
548 by a rich, dense network of cortico-cortical, commissural and associational long-range axonal projections.
549 In experimental animals, L1 consists exclusively of a heterogeneous population of GABAergic interneurons
550 providing feedforward and feedback inhibition (see for example Jiang et al. 2013; Boldog et al. 2018;
551 Obermayer et al. 2018; Kwon et al. 2019; reviewed by Huang et al. 2024) providing signals to the terminal
552 tuft dendrites of pyramidal neurons (reviewed by Schumann et al. 2021). In humans, there is a persistent
553 subpopulation of CR cells that provides excitation due to their long-range transcolumnar axonal projection
554 to the terminal tuft dendrites (Anstötz et al. 2014), but may also control the population of GABAergic
555 interneurons, which is as to date unknown.

556 Thus, L1 in the human neocortex is a central locus of neocortical associations controlled by excitation
557 and distinct types of inhibition (reviewed by Hartung and Letzkus 2021; Schumann et al. 2021). In summary,
558 the population of GABAergic interneurons in L1 provides inhibition and disinhibition to pyramidal terminal
559 tuft dendrites by either shunting or promoting the generation of Ca^{2+}- and Na^{+}-spikes by temporal and spatial
560 coincidence detection. Thus, the circuitry of L1, including the persistent subpopulation of excitatory CR-
561 cells; is an important and fundamental 'integrator' of the neocortex sampling bottom-up information within
562 or even across cortical columns, but also act as a 'filter' or 'discriminator' but also as an 'amplifier' in the
563 information processing of the neocortex.

564 Hence, L1 of the neocortex can be regarded as a complex and intricate layer of the brain serving different
565 functions, for example to gate inputs, convey expectations and context as well as mediate states of
566 consciousness, attention, cross-modal interactions, sensory perception, and learning (Bastos et al. 2012;
567 Heeger 2017; Zagha 2020; reviewed by Gilbert and Li 2013). There is evidence that L1 processing,
568 particularly Ca^{2+} signals in the distal apical dendrite, is important for sensory perception (Xu et al. 2012).

569 Recent studies have revealed that L1 plays a crucial role in top-down attentional processes, aiding in
570 directing attention, based on our goals and intentions (Hartung and Letzkus 2021; Schuman et al. 2021).
571 Additionally, L1 is implicated in the formation and consolidation of long-term memories with disruptions
572 in L1 function associated with memory deficits. Furthermore, L1 contributes to brain plasticity, which
573 involves the brain's ability to change and adapt in response to experience, including the process by which
574 synapses alter in strength and number (reviewed by Hartung and Letzkus 2021; Schuman et al. 2021).
575 Overall, L1 of the neocortex is a complex and fascinating layer of the brain that is involved in a wide
576 range of cognitive processes and brain plasticity. Since this is relayed by a network of SBs in L1, their
577 composition and connections play a pivotal role, in columnar information processing.
578
579
580 **Material and Methods**
581 Human brain tissue sampling during epilepsy surgery have been provided by Dr. med. Dorothea Miller, PD
582 Dr. med. Marec von Lehe, Department of Neurosurgery, Knappschaftschafts/Universitäts-Krankenhaus
583 Bochum and were approved by the Ethical Committees of the Rheinische Friedrich-Wilhelms-
584 University/University Hospital Bonn (Ethical votum of the Medical Faculty to Prof. Dr. med. Johannes
585 Schramm and Prof. Dr. rer. nat. Joachim Lübke, Nr. 146/11), the University of Bochum (Ethical votum of
586 the Medical Faculty to PD Dr. med. Marec von Lehe and Prof. Dr. rer. nat. Joachim Lübke, Reg. No. 5190-
587 14-15; and renewed Ethical votum of the Medical Faculty to Dr. med. Dorothea Miller and Prof. Dr. rer.
588 nat. Joachim Lübke, Reg. No. 17–6199-BR). The consent of the patients was obtained by written and signed
589 statements, and all further experimental procedures were approved by the same Ethical Committees cited
590 above, and the EU directive (2015/565/EC and 2015/566/EC) concerning working with human tissue used
591 for experimental and scientific purposes. All subsequent experimental procedures were approved by the
592 Research Committee of the Research Centre Jülich GmbH.
593
594

595 **Fixation and tissue processing for TEM**

596 Tissue samples from the human TLN were after their removal prepared and embedded for conventional
597 TEM and EM tomography analysis. All neocortical access tissues were obtained from patients suffering
598 from drug-resistant temporal lobe epilepsy (1 male and 3 female, 24-65 years in age for L1, and additionally
599 2 male, and 3 female, 25-63 in age for L4; see also Supplemental Table 2).

600 The pre-surgical work-up comprised high-resolution magnetic resonance imaging together with long-
601 term video-electro-encephalography (EEG)-monitoring and electrophysiology using multiple electrode
602 recordings. In all cases, the circumscribed epileptic focus was in the hippocampus proper, but not in the
603 neocortical access regions of the TL.

604 During epilepsy surgery, blocks of both non-affected, non-epileptic and epileptic neocortical access
605 tissue samples (see Supplemental Table 2) were resected and part of the tissue samples were further
606 histologically examined by neuropathologists. The non-affected non-epileptic neocortical access tissue
607 samples were always taken far from the epileptic focus and may thus be regarded as non-epileptic as also
608 demonstrated by functional studies using the same experimental approach (Mohan et al. 2015; Molnár et
609 al. 2016; Seeman et al. 2018; Obermayer et al. 2018; reviewed by Mansvelder et al. 2019). These studies
610 have clearly demonstrated that neocortical access tissue samples taken from epilepsy surgery do not differ
611 in electrophysiological properties and synaptic physiology when compared with acute slice preparations in
612 experimental animals.

613 So-called 'post mortem' tissue was not used in this study since the ultrastructural quality (preservation)
614 of such material is not suitable enough for fine-scale high-resolution EM, due to severe distortions of
615 relevant structural features, e.g. fragmentation and lysis of membranes of SBs, PreAZs, PSDs and SVs that
616 are required for the generation of quantitative 3D-models of SBs and their target structures (Lübke, personal
617 observation). However, under certain conditions also 'post mortem' tissue samples can be used
618 (Dominguez-Alvaro et al. 2019, 2921; Cano-Astorga et al. 2021, 2023).

619 Inter-individual differences were found in the structural and synaptic parameters analyzed as shown by
620 the box plots (Supplemental Figures 1, 2; Source Data 02).

621 Other recent studies using the same experimental approach neglected and thus discarded the effect of the
622 pharmacological treatments and disease condition (Alonso-Nanclares et al. 2008; Testa Silva et al. 2014;
623 Mohan et al. 2015; Molnár et al. 2015; Seeman et al. 2018; Dominguez-Alvaro et al. 2019; Yakoubi et al.
624 2019a, b; Schmuhl-Giesen et al. 2022).

625 For the study of L1 in the human TLN, blocks of neocortical access tissue were sampled from the
626 temporo-lateral or temporo-basal regions of the inferior temporal gyrus and the gyrus medialis. Immediately
627 after their removal during epilepsy surgery, biopsy samples of the TLN were immersion-fixed in ice-cold
628 4% paraformaldehyde and 2.5% glutaraldehyde diluted in 0.1 M phosphate buffer (PB, pH 7.4) for 24-72
629 hrs at 4°C. The fixative was replaced by fresh fixative after 2 hrs and changed twice during the subsequent
630 fixation period. Prior to vibratome sectioning, brain tissue samples were thoroughly rinsed in ice-cold PB
631 and afterwards embedded in 5% Agar-Agar (Sigma, Munich, Germany) diluted in PB.

632 Neocortical tissue blocks were cut in the coronal plane through the TLN with a Vibratome VT 1000S (Leica
633 Microsystems GmbH, Wetzlar, Germany) into 150-200 µm thick sections, collected in ice-cold PB, and
634 washed again several times in PB. Afterwards they were transferred to 0.5-1% PB-sucrose buffered
635 Osmium tetroxide (OsO_4, 300 mOsm, pH 7.4; Sigma, Munich, Germany) for 60-90 min. After visual
636 inspection to check for the quality of post-fixation, sections were thoroughly washed several times in PB
637 and left overnight at 4°C in PB. The next day they were dehydrated in an ascending series of ethanol starting
638 at 20%, 30%, 50%, 60%, 70%, 80%, 90%, 95% to absolute ethanol (15 min for each step and absolute
639 ethanol, 30 min twice), followed by a brief incubation in propylene oxide (2 min twice; Fluka, Neu-Ulm,
640 Germany). Sections were then transferred into a mixture of propylene oxide and Durcupan™ resin (2:1, 1:1
641 for 1h each; Fluka, Neu-Ulm, Germany) and stored overnight in pure resin. The next day, sections were flat
642 embedded on coated glass slides in fresh Durcupan™, coverslipped with Acla™ foils and polymerized at
643 60°C for 2 days.

644

645

646

647 **Semi- and ultrathin sectioning**

648 After light microscopic (LM) inspection, a tissue block containing the region of interest (ROI) was glued
649 on a pre-polymerized block and trimmed down. Semithin sections were cut with a Leica UltracutS
650 ultramicrotome (Leica Microsystems, Vienna, Austria), with a Histo-Diamond knife (Fa. Diatome, Nidau,
651 Switzerland). Afterwards they were briefly stained with methylene-blue (Sigma-Aldrich Chemie GmbH,
652 Taufkirchen, Germany) to identify the cortical layers, particularly L1 and underlying L2, examined and
653 documented using a motorized Olympus BX61 microscope equipped with the Olympus CellSense analysis
654 hard- and software (Olympus GmbH, Hamburg, Germany). All images were stored in a database until
655 further use.

656 After further trimming of the block to its final size, serial ultrathin sections (50 ± 5 nm thickness; silver
657 to silver gray interference contrast) were cut with a Leica UltracutS ultramicrotome through the determined
658 ROI of L1a and L1b, respectively. Ultrathin sections in serial sequence were collected on pioloform-coated
659 slot copper grids (Plano, Wetzlar, Germany). Prior to EM examination, sections were stained with 5%
660 aqueous uranyl acetate or Uranyless (Science Services, Munich, Germany) for 15–20 min and lead citrate
661 for 3–5 min according to Reynolds (1963) to enhance the contrast of biological membranes at the EM
662 level. In each series of ultrathin sections, two or three ROIs throughout L1a or L1b were chosen, and
663 photographed at a final EM magnification of 8000x with a Zeiss Libra 120 (Fa. Zeiss, Oberkochen,
664 Germany) equipped with a Proscan 2K digital camera (Fa. Tröndle, Moorenweis, Germany) using the
665 ImageSP software and its panorama function (Fa. Tröndle, Moorenweis, Germany). In addition, interesting
666 details of synaptic structures in L1a and L1b were taken at various EM magnifications.

667 All images were stored in a database until further use. Selected EM images for publication were further
668 edited using Adobe Photoshop™ and Adobe Illustrator™ software.

669

670 **3D-volume reconstructions and quantitative analysis of SBs**

671 EM panorama images composing each series were imported, stacked, and aligned in the reconstruction
672 software OpenCAR (Contour Alignment Reconstruction; for details see Sätzler et al. 2002). The main goal

673 of this study was to quantify several structural and synaptic parameters representing structural correlates of
674 synaptic transmission and plasticity. Excitatory SBs were characterized by large round SVs and prominent
675 PreAZs and PSDs in contrast to putative GABAergic terminals that have smaller, more oval-shaped SVs,
676 and a thin or no PSDs.

677 The following structural parameters were analyzed in randomly selected excitatory SBs: (1) surface area
678 and volume of SBs; (2) volume of mitochondria; (3) surface area of PreAZs (Dufour et al. 2016) and PSDs;
679 two apposed membrane specializations separated by the synaptic cleft; (4) number and diameter of clear
680 SVs and dense-core vesicles (DCVs); and (5) distance of individual SVs from the PreAZ for the structural
681 definition of the RRP, RP, and resting pool. 3D-volume reconstructions were generated by drawing contour
682 lines on the structures of interest in each panorama image within a series of images until a given structure
683 was completed. Presynaptic SBs, their mitochondria as well as their postsynaptic target structures were
684 outlined on the outer edge of their membranes, using closed contour lines throughout the entire stack within
685 a series. A SB was considered completely captured, when it was possible to follow the axon in both
686 directions through the entire series (*en passant* SBs) or the enlargement of the axon leading to an
687 endterminal SB. The beginning of a synaptic terminal was defined by the typical widening of the axon and
688 the abrupt occurrence of a pool of SVs.

689 The PreAZs and PSDs were regarded as complete when their perimeters were entirely reconstructed in
690 a series of ultrathin sections. The surface areas of the PreAZ and PSD were computed separately by first
691 generating a 3D surface model of the SB. The PreAZ was then measured by extracting this area from the
692 reconstructed presynaptic bouton membrane that was covered by this membrane specialization. Hence, the
693 length (L) of the PreAZ (L PreAZ) and the surface area (SA) of the PreAZ (SA PreAZ) is already known.

694 The size of the PSD opposing the PreAZ was estimated under the following assumptions: (1) both
695 membrane specializations, PreAZ and PSD run parallel to each other at the pre- and postsynaptic apposition
696 zone; (2) for both membrane specializations a contour line was drawn determining their actual length (L
697 PreAZ and L PSD). Hence, the surface area of the PSD (SA PSD) is estimated by the following equation:

698 $$\mathbf{SA\ PSD = SA\ PreAZ * L\ PSD / L\ PreAZ}$$

699 which is the perimeter ratio between the outlines of the PSD to that of the synaptic contact.
700 The synaptic cleft width was measured because of its importance for the transient temporal and spatial
701 increase of the glutamate concentration, reversible binding of glutamate to appropriate glutamate receptors
702 and eventual up-take and diffusion of glutamate out of the synaptic cleft. To a large extent, these processes
703 are governed by the geometry of this structure and the shape and size of the PreAZs and PSDs.
704 All SVs were marked throughout each SB, their diameters were individually measured and their
705 distances to the PreAZ were automatically detected using an algorithm implemented in OpenCAR (minimal
706 distance between each SV membrane to the contour line of the PreAZ) throughout the SB in every single
707 image of the series. Large DCVs were only counted in the image where they appeared largest. To avoid
708 double counts, only clear ring-like structures were counted as SVs. However, SVs might be missed in
709 densely packed regions, because ring-like traces may partly overlap. This effect may counteract any double
710 counts. Based on the small extent and the partially counteracting nature of this effect, the numbers of small
711 clear vesicles reported in this study remained uncorrected (Yakoubi et al. 2019a, b, Schmuhl-Giesen et al.
712 2022).
713 In this work, aldehyde fixation was used that is thought to induce tissue shrinkage thereby biasing
714 structural quantification (Eyre et al. 2007; Korogod et al. 2015). A direct comparison of structural
715 parameters obtained from either aldehyde or cryo-fixed and substituted tissue samples (Korogod et al.
716 2015), showed differences in cortical thickness (~16% larger in cryo-fixed material), in the volume of
717 extracellular space (~6-fold larger in cryo-fixed material), a slight increase in glial volume and overall
718 density of synaptic contacts (~14% in cryo-fixed material), but no significant differences in neuronal
719 structures such as axons, dendrites and SV diameter.
720 In the structural and synaptic parameters as estimated here, no significant difference was found for SB
721 size and other synaptic subelements such as mitochondria, active zones (AZs) and SVs when compared
722 with other studies (Zhao et al. 2012a, b). Therefore, no corrections for shrinkage were applied and we are
723 thus convinced that the synaptic parameters reported here are accurate and can be directly used for detailed
724 computational models. In addition, large-scale preservation for ultrastructural analysis will therefore

725 continue to rely on chemical fixation approaches, due to the limited preservation of the ultrastructure in
726 cryo-fixed material as stated in Korogod and co-workers (2015).
727
728 **Golgi-Cox impregnation of biopsy material in L4 of the human TLN**
729 To get an impression about the neuronal organization of the human TLN, four tissue blocks were processed
730 with the Golgi-Cox impregnation technique using the commercially available Hito Golgi-Cox OptimStain
731 kit (Hitobiotec Corp, Kingsport, TE, USA). After removal of the biopsy samples, tissues were briefly rinsed
732 twice in double distilled water (dd H_2O), and then transferred into impregnation solution 1 overnight at
733 room temperature. The next day, tissue samples were incubated in fresh impregnation solution 1 and stored
734 for 14 days in the dark at room temperature. The samples blocks were then transferred in solution 3 and
735 kept in the dark at room temperature for one day. Thereafter, they were placed into fresh solution 3 in the
736 dark at room temperature for 6 additional days. Then, solution 3 was exchanged and samples were stored
737 at 4°C in the dark overnight. Tissue blocks were embedded in 5% agarose (Carl Roth, Karlsruhe, Germany)
738 diluted in ddH_2O, and sectioned with a vibratome in the coronal plane at 100–250 µm thickness and then
739 transferred to ddH_2O.
740 After careful removal of the agarose, free-floating sections were incubated into solution 3 for 2–3 min
741 in the dark at room temperature, and right after placed into ddH_2O, washed several times and stored
742 overnight. Afterwards, they were rinsed twice in ddH_2O for 4 min each, and dehydrated in 50%, 70% and
743 95% ethanol for 5 min each, then transferred into absolute ethanol (3 × 5 min), defatted in xylene, embedded
744 in Eukitt™ (Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany), finally coverslipped and air-dried.
745 Afterwards, sections were examined and imaged with an Olympus BX 61 LM equipped with the CellSense
746 software package (Olympus, Hamburg, Germany) at various magnifications and images were stored in a
747 database until further use.
748
749
750

751 **Stereological estimation of the density of L1 synaptic contacts in the human TLN**

752 The density of synaptic complexes, composed either between an SB with a dendrite or spine, in a given
753 volume is a valuable parameter to assess the structural and functional changes in the brain, which are linked
754 to the age, pathological or experimental conditions (Rakic et al. 1994; DeFelipe et al. 1999). The density
755 of synaptic contacts was unbiasedly estimated in L1, separated for L1a and L1b, from four patients,
756 respectively (Supplemental Table 2; Source Data 01) using the physical dissector technique (Mayhew 1996;
757 Fiala and Harris 2001) by counting the synaptic complexes in a virtual volume generated by two adjacent
758 ultrathin sections that is the dissector: the reference section and the look-up section. Here, counting was
759 performed using FIJI (Schindelin et al. 2012) on a stack of 20 aligned serial electron micrographs for each
760 patient taken from the series of ultrathin sections used for the 3D-volume reconstructions of SBs in L1. An
761 unbiased counting frame was first set and synaptic contacts to be considered (counted) are the one present
762 in the reference section only and meeting the following criteria: presence of a PreAZ and a prominent or
763 thin PSD separated by a synaptic cleft and SVs in the presynaptic terminal. Care was taken to distinguish
764 between excitatory and inhibitory synaptic contacts, as well as the postsynaptic target structures (dendritic
765 shafts or spines). Finally, the density of synaptic contacts (*Nv*) per 1 mm^3 was calculated using the formula
766 below:

767 $$\mathbf{Nv=\sum Qd/\sum Vd}$$

768 where *Qd* is the number of synaptic contacts per dissector and Vd is the volume of the dissector given by:
769 Number of dissectors x frame area x section thickness.

770

771 **TEM tomography of L1 SBs in the human TLN**

772 EM tomography was performed on 200-300 nm thick sections cut from blocks prepared for serial ultrathin
773 sectioning as described above (Table 2; Source Data 03). Sections were mounted on pioloform-coated line
774 copper grids and were counterstained with uranyl acetate and lead citrate following a slightly modified
775 staining protocol as described by Reynolds (1963).

776 Subsequently, sections were examined with a JEOL JEM 1400Plus, operating at 120 kV and equipped with
777 a 4096×4096 pixels CMOS camera (TemCam-F416, TVIPS, Gauting, Germany). Tilt-series were acquired
778 automatically over an angular range of −60° to +60° at 1°-degree increments using Serial EM (Mastronarde
779 2005). Stack alignment and reconstruction by filtered backprojection were carried out using the software
780 package iMOD (Kremer et al. 1996). Final reconstructions were ultimately filtered using a median filter
781 with a window size of 3 pixels. Tilt-series were stored as .tif files and were further processed using the
782 freely available software Imod 4.9.12 and ImageJ (ImageJ, RRID:SCR-0033070). In each tilt-series so-
783 called 'docked' SVs identified by their fusion with the PreAZ or as omega-shaped body that already
784 released a quantum of neurotransmitter were counted separately for L1a and L1b and for their target
785 structures, dendritic shafts or spines in randomly selected SBs.

786 To determine possible differences to the values obtained by the 3D-reconstructions, in the same tilt-series
787 binned at 24 nm (section thickness) the diameter of SVs was measured randomly by using OpenCAR as
788 described above. To avoid double counts and underestimations of the diameter, the ring-shaped structure of
789 an SV was compared on each tilted-slice and only marked if it appears largest. In 18 tilt-series each consisting
790 of 9-14 tilt-slices, randomly selected SBs were investigated (L1a: 8 tilt series; 16 SBs with a total of 2094
791 SVs; L1b: 10 tilt series; 22 SBs with a total of 3740 SVs).

792

793 **Quantitative analysis of the astrocytic coverage**

794 Although methodologically associated with the possibility of a certain degree of error, astrocytic processes
795 around synaptic complexes were mainly identified by their relatively clear cytoplasm compared to synaptic
796 boutons or dendritic profiles; in addition by their irregular, stellate shape, by the presence of glycogen
797 granules and bundles of intermediate filaments (Peters et al. 1991; Ventura and Harris 1999). With greater
798 accuracy, astrocytic processes can be identified with immunolabeling, but this leads to poorer tissue
799 preservation and resolution of cellular structures such as AZs. Among studies in rats (e.g. Rollenhagen et
800 al. 2015; 2018) in a previous study on the human TLN (Yakoubi et al. 2019a) immunohistochemistry against
801 glutamine synthetase, a key enzyme in astrocytes, was carried out. Since similar results were obtained

802 without immunohistochemistry when identifying astrocytic processes around AZs, immunohistochemistry
803 was omitted in the series of follow-up studies on the human TLN.
804 To quantify the volume contribution of astrocytic processes in the human neocortex, the interactive
805 software ImageJ (Schneider et al. 2012) was used. The first, the middle, and the last images of an individual
806 EM series were used for a further quantitative volumetric analysis (Source Data 04, sheet 01). In each section
807 of the same series used for the 3D-volume reconstructions, a grid (grid size 1 × 1 μm^2) was placed over the
808 EM image, and in each square, the abundance of fine astrocytic processes was documented throughout these
809 images and averaged. Using the Cavalieri method [Unbiased Stereology: Three-Dimensional Measurement
810 in Microscopy (Advanced Methods) Paperback-January 7, 2005, by Vyvyan Howard Matthew Reed], the
811 (absolute) volume contribution of astrocytic processes was determined according to the Cavalieri estimator:
812 $$\mathbf{V=a(p)\times\Sigma P\times t}$$
813 where a(p) is the size of one square (0.8 × 0.8 μm2), P is the number of squares counted, and t is the
814 thickness of the slice.
815 In addition, in consecutive sections spanning the complete AZ , the astrocytic coverage was evaluated
816 under the following criteria: fine astrocytic processes were observed on 1) both sides of the AZ reaching as
817 far as the synaptic cleft, 2) only one side of the AZ was covered by fine astrocytic processes and 3) AZs
818 without astrocytic processes (Source Data 04, sheet 02). Values obtained for L1 (L1a: n = 3 patients; L1b:
819 3 patients) either for SBs terminating on dendritic spines (L1a: n = 128; L1b: n=126) or shafts (L1a: n= 48;
820 L1b: n = 44) were compared to L4 (n = 5 patients; dendritic spines n=93; dendritic shafts n=41). Layer 4
821 was chosen because the volumetric analysis showed the highest portion of astrocytic processes in this layer
822 compared to all other cortical layers. All values are given as percentages.
823
824 **Statistical analysis**
825 The mean value ± standard deviation (SD), the median with the interquartile range (IQR), the coefficient
826 of variation (CV), skewness, variance, and the coefficient of correlation (R^2) were given for each structural
827 parameter analyzed. The p-value was considered significant only if $p < 0.05$. Box- and Violin plots (Plotly

4.0.0 https://chart-studio.plotly.com) were generated to investigate inter-individual differences for each patient and structural parameter (Supplemental Figure 1, 2; Source Data 02).

To test for significant differences, the non-parametric Kruskal-Wallis H-test with a subsequent Mann-Whitney u-test analysis was performed, using PAST 4.02 (Hammer et al. 2001). Correlation graphs between several structural parameters were then generated (Figure 4). The R^2-values were interpreted as follows: 0, no linear correlation; 0-0.5, weak linear correlation; 0.5-0.8, good linear correlation; and 0.8-1.0, strong linear correlation. Furthermore, a freely available Fisher's *r*-to-*z*-transformation calculator (Fisher's z) was used to test for differences in R^2 between L1a and L1b (p-value < 0.05).

For statistical comparison with other layers of the human TLN, L1a and L1b were grouped together as L1.

Parts of the data acquisition were carried out in the context of a PhD Thesis (Sadeghi Dastjerdi A. 2024).Values obtained in the present study for L1 were compared partially to those obtained for L2, L3 (Girwert Ch., PhD Thesis in preparation), L4, L5 (Yakoubi et al. 2019a, b; Yakoubi R., PhD Thesis 2023) and L6 (Schmuhl-Giesen et al. 2022).

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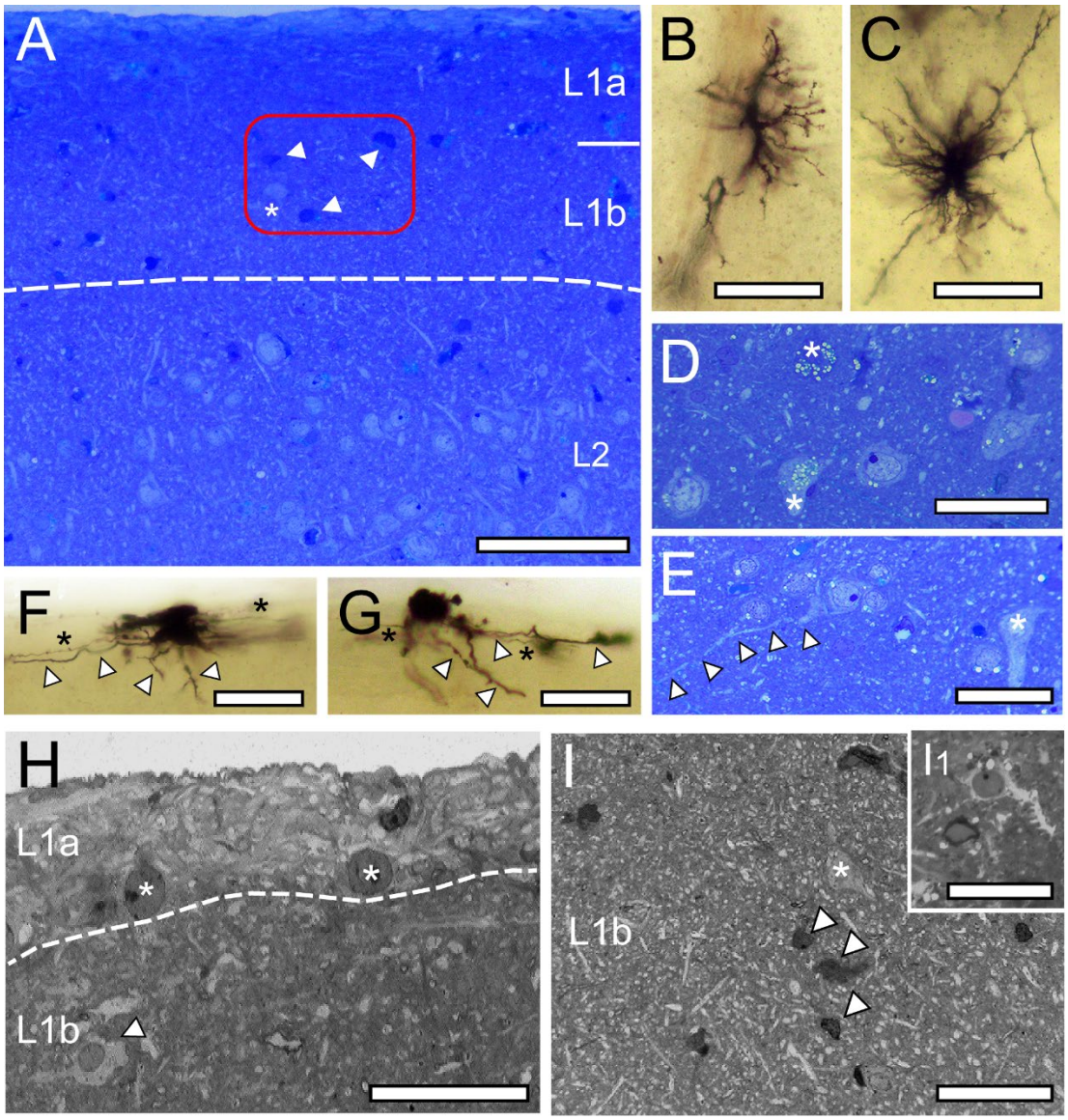
Authors contributions

Conceptualization, J.H.R.L. and A.R.; Methodology, J.H.R.L., A.R. and A.S.D.; Investigation, J.H.R.L., A.R. and A.S.D.; Writing – Original Draft, J.H.R.L., A.R. and A.S.D.; Writing – Review & Editing, J.H.R.L., A.R. and A.S.D.; Funding Acquisition, J.H.R.L. Resources, J.H.R.L.; Supervision, J.H.R.L. and A.R.

Declaration of interests

The authors disclose any financial or other interests related to the submitted work that could affect, or have the perception of affecting, the author's objectivity, or could influence, or have the perception of influencing, the content of the article.

1211 **Figures and legends**



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1213 **Figure 1: L1 and L2 in the human TLN as revealed in methylene-blue stained semithin sections, Golgi-**
1214 **impregnation and low power EM.**
1215 **A,** Methylene-blue stained semithin section of L1a, L1b, and L2. The cell sparse zone contains degenerating
1216 neurons (dark appearance, arrowheads in the framed area) and a cell body (asterisk in the framed area). Note
1217 the sudden increase in the density of neurons at the L1b/L2 border. Scale bar 500 µm. **B, C,** Golgi-
1218 impregnated radial astrocytes in L1a (B) and L1b (C). Scale bars 50 µm. **D,** Group of neurons in L1b
1219 containing lipofuscin granules of various shape and size (asterisks) most prominent in two of the neurons.
1220 Scale bar 50 µm. **E,** Putative CR-cell identifiable by its long horizontally oriented dendrite (marked by
1221 arrowheads) and an inverted putative GABAergic interneuron (asterisk) in L1b. Scale bar 50 µm. **F, G,**
1222 Golgi-impregnated CR-Cells in L1a with the characteristic horizontal orientation of dendrites (arrowheads)
1223 and axons (asterisks). Scale bars 50 µm. **H,** EM micrograph of L1a underneath the pial surface containing
1224 two putative CR-cells (asterisks). Note the abrupt transition (indicated by the dashed line) to the neuropil of
1225 L1b, and a reactive microglial cell (arrowhead), and dendrites and synapses. Scale bar 25 µm**. I,** EM
1226 micrograph of L1b containing thousands of dendritic profiles, a putative GABAergic interneuron (asterisk)
1227 but also degenerating neurons (arrowheads) and a reactive microglia (I1) Scale bar 50 µm**.**

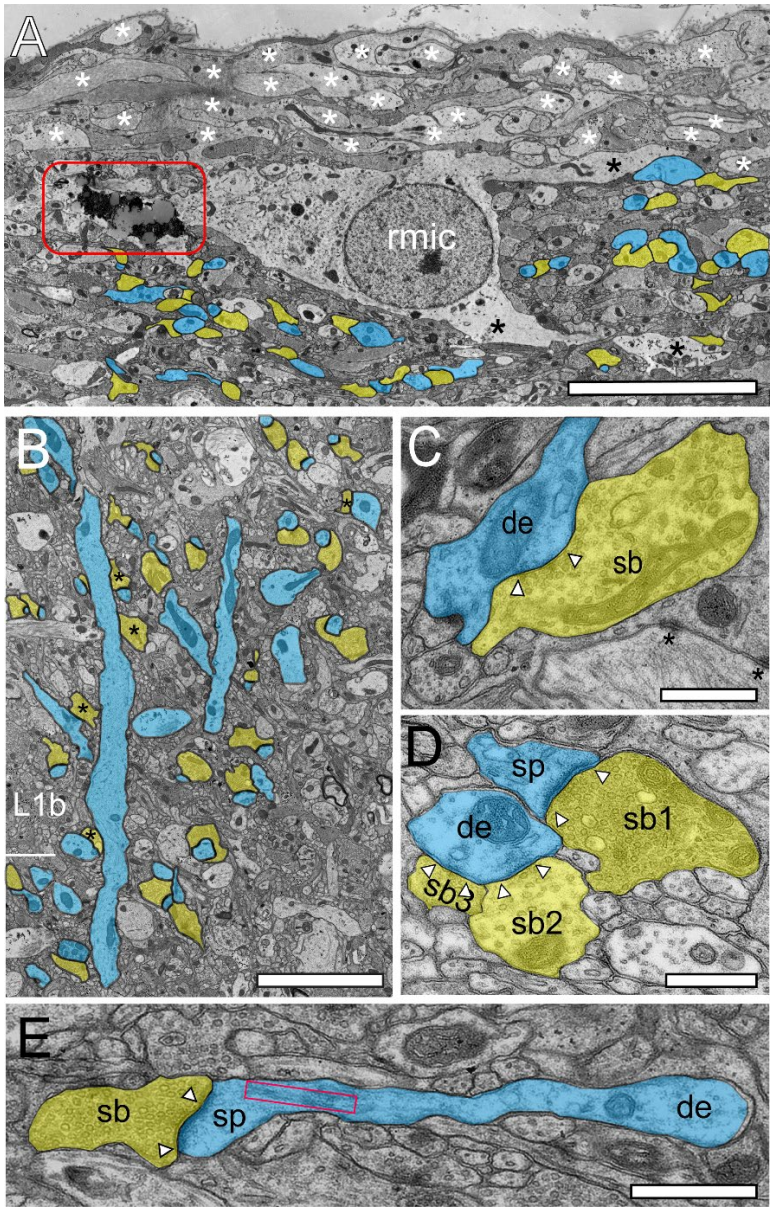


Figure 2: Structural and synaptic organization of L1 in the human TLN as revealed by EM.
A, Superficial part of L1a composed of a dense network of fine astrocytic processes (asterisks). In the reactive microglial cell (rmic) so-called lipofuscin granules (framed area) were observed. Below the astrocytic network synaptic complexes gradually increased (highlighted in blue for target structures, yellow for SBs). Scale bar 10 µm. **B,** Two apical dendrites (blue) traversing L2 with beginning terminal tuft dendrites in L2 and L1 contacted by SBs (transparent yellow); some of which are putatively GABAergic terminating on the dendritic shaft (asterisks). Scale bar 2.5 µm. **C,** Large GABAergic SB (sb, transparent yellow) identifiable by the small-sized ovoid SVs and the less prominent PSD (arrowheads) synapsing on a dendritic shaft (de, transparent blue) in L1b. **D,** Three SBs in L1a (sb1-sb3, transparent yellow) located on a dendritic spine (sp, transparent blue) and a neighboring dendritic shaft (sh, transparent blue). Note that sb1 is excitatory as indicated by the larger round SVs with a prominent AZ whereas sb2 and sb3 are putative inhibitory terminals identified by smaller ovoid SVs and the lack of a prominent PSD. **E,** Elongated spine (sp) emerging from a small caliber dendrite (de, transparent blue) receiving input from a small-sized SB (sb, transparent yellow). Note the large, elongated spine apparatus (framed area). Scale bar in C-E 0.5 µm. In graphs C-E AZs are marked by arrowheads.

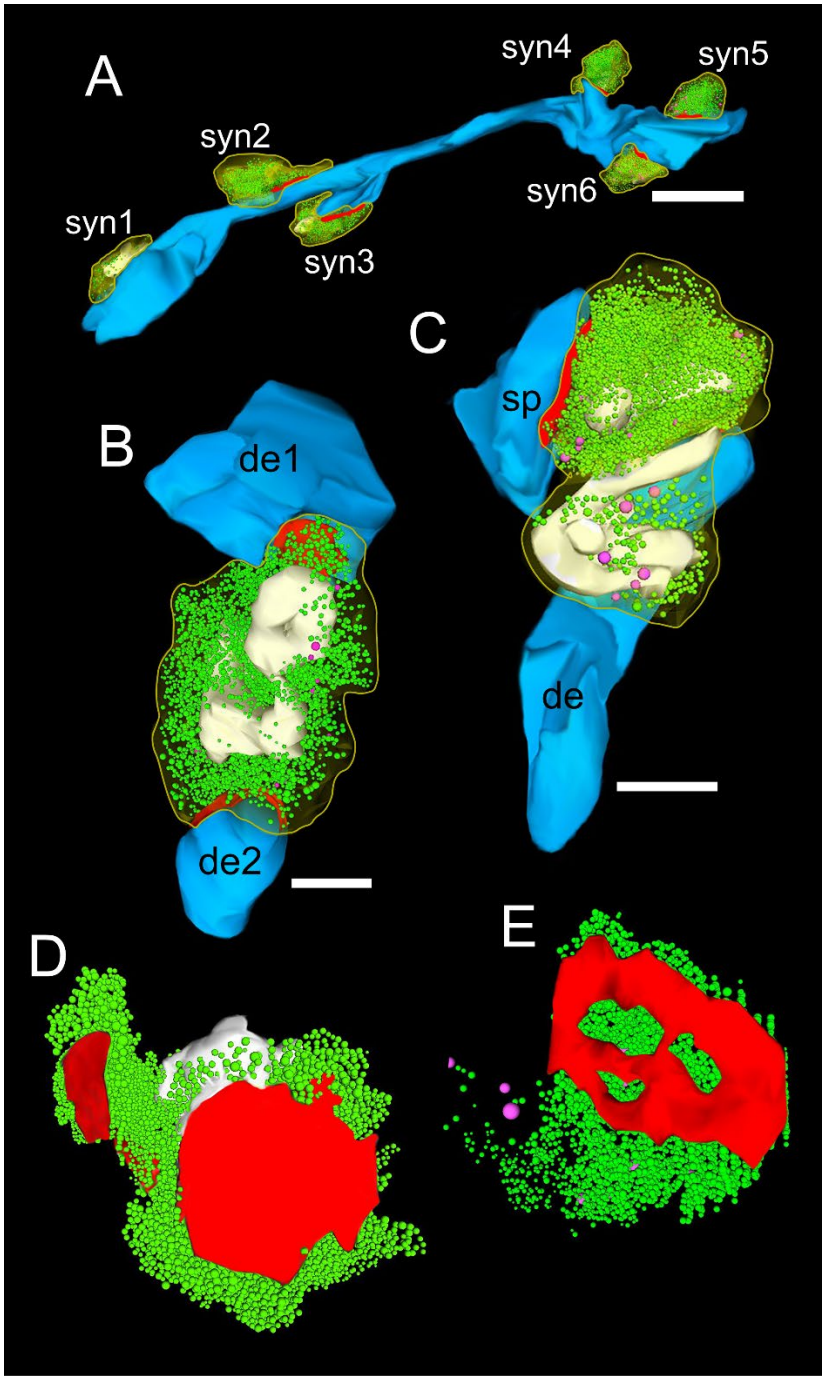


Figure 3: 3D-volume reconstructions of SBs and vesicle pools in L1a and L1b of the human TLN. **A-C,** Here, all SBs are outlined by a yellow contour. Subelements: PreAZs (red), SVs (green), DCVs (magenta), mitochondria (white). The postsynaptic target structures are given in blue **A,** Six SBs (sb1-sb6) terminating at different locations on a dendritic segment in L1b, two of which (syn3 and syn4) were located on spines, the remaining on the dendritic shaft. Scale bar 1 µm. **B,** Large SB terminating on two dendritic segments of different shape and size (de1, de2) in L1a. **C,** SB synapsing on a dendrite (de) and spine (sp) in L1b containing several mitochondria associated with the pool of SVs. **D, E,** 3D-volume reconstructions of the total pools of SVs (green dots). Note the different shape and size of the PreAZs (red) with either a non-perforated macular (D) or perforated (E) appearance and the comparably large total pool size. Note the large DCVs intermingled with the pool of SVs.

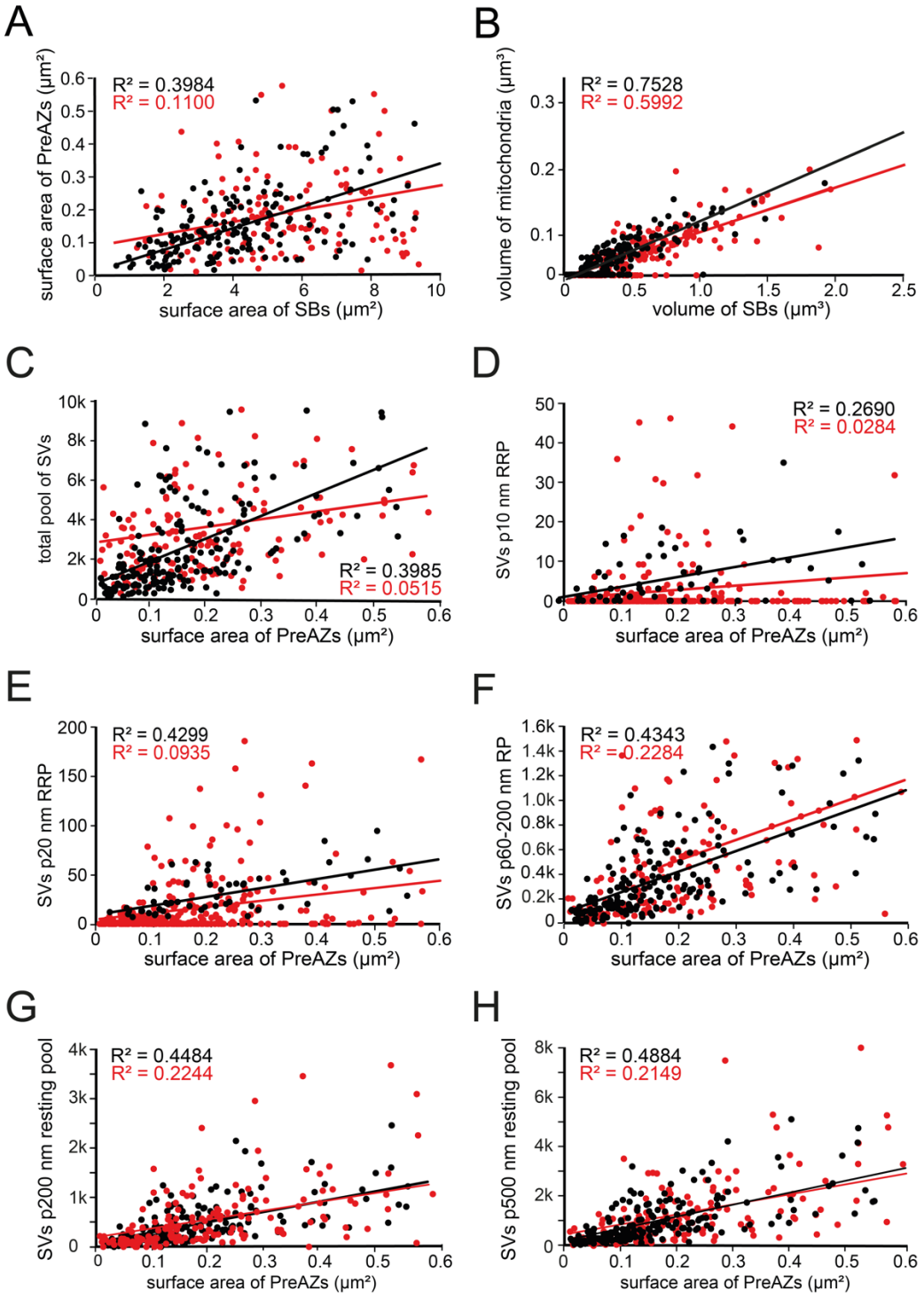


Figure 4: Correlation plots for structural and synaptic parameters characterizing L1 SBs. Correlation plots showing the strength of correlations between structural and synaptic parameters (Source Data 02). Correlations dots and regression lines for L1a are given in black and that for L1b in red. **A,** Surface area of SBs vs. surface area of PreAZs; **B,** Volume of SBs vs. volume of mitochondria; **C,** Surface area of PreAZs vs. total pool of SVs; **D,** Surface area of PreAZs vs. p10 nm RRP; **E,** Surface area of PreAZs vs. p20 nm RRP; **F,** Surface area of PreAZs vs. p60-p200 nm RP; **G,** Surface area of PreAZs vs. p200 nm resting pool; **H,** Surface area of PreAZs vs. p500 nm resting pool.

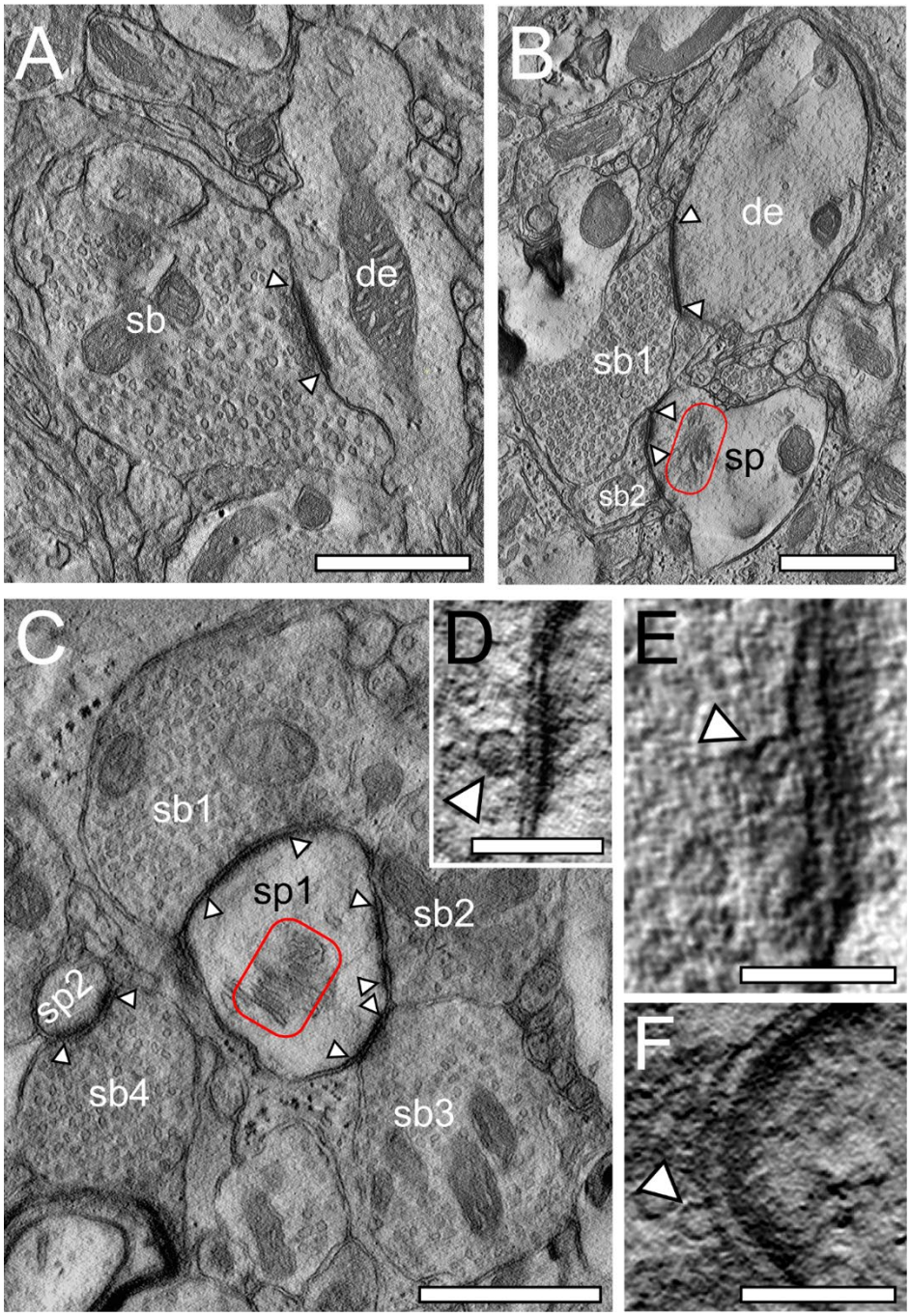


Figure 5: EM tomography of SBs in L1a and L1b in the human TLN.
A, Large SB (sb) terminating with a single AZ (arrowheads) on a terminal tuft dendritic shaft (de) in L1a. **B,** Two adjacent SBs synapsing either (sb1) on a thick dendritic segment (de) or (sb2) on a stubby spine (sp) with a prominent spine apparatus (framed area) in L1b. Scale bars 1 µm. **C,** Large mushroom (sp1) and a small spine head (sp2) receiving input from three SBs (sb1, sb2, sb3) and a single SB (sb4) in L1b. Note the large AZs (arrowheads) and the prominent spine apparatus (red framed area) in the mushroom spine. Scale bar 0.5 µm. **D, E, F,** High-power images of 'docked' SVs (arrowheads) taken from a tilt-series through an individual PreAZ at a L1a spine SB. Note the so-called omega-shaped bodies in (E, F) pointing to the already opening and release of glutamate quanta. Scale bars 0.1 µm.

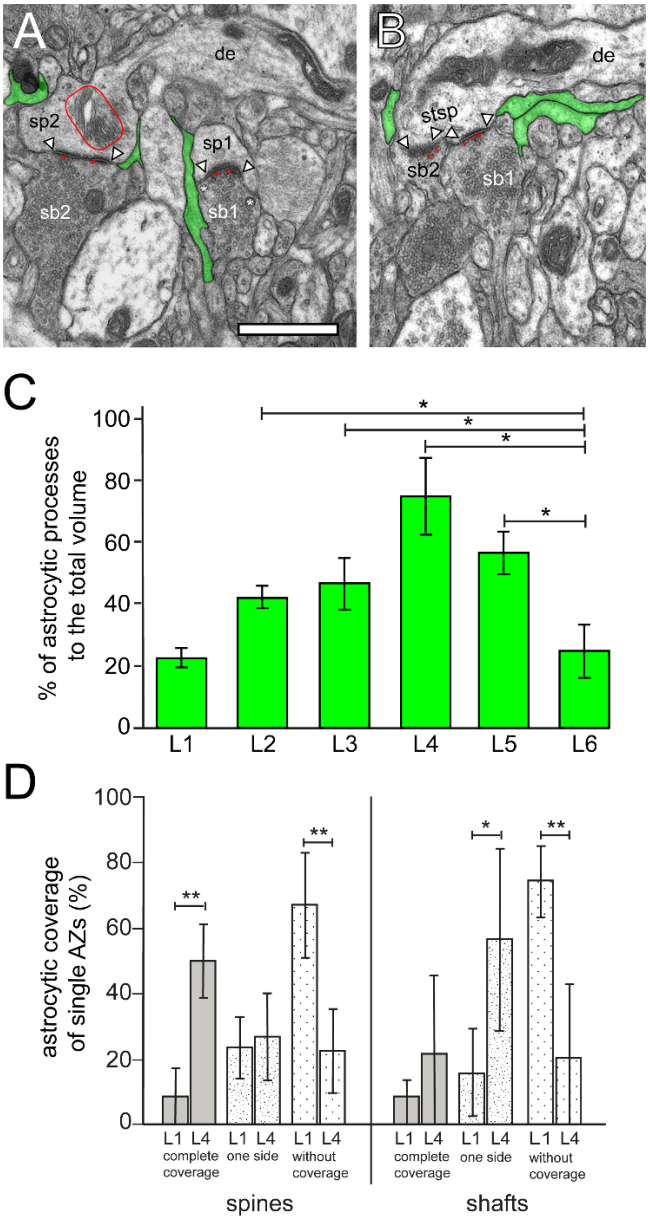


Figure 6: Astrocytic coverage of synaptic complexes in the human TLN.
A, EM micrograph of two adjacent SBs (sb1, sb2) terminating on two spines (sp1, sp2) in L1a. Sp2 contained a prominent spine apparatus (framed area), sb1 two DCVs (asterisks). Note that both synaptic complexes were only partially ensheathed by fine astrocytic processes (transparent green) reaching the AZs only on one side. In both synaptic complexes two 'docked' SVs (transparent red) at the PreAZs were found. Scale 0.25 µm. **B,** Stubby spine (stsp) receiving two SBs (sb1, sb2) in L1b. Here, fine astrocytic processes (transparent green) were found close to the two synaptic complexes but never reached the synaptic cleft. Note also the 'docked' SVs (transparent red) pointing to multivesicular release. In both images the AZs are marked by arrowheads. Scale bar 0.25 µm. **C,** Bar histogram showing the percentage (mean ± SD) of the volumetric fraction of astrocytic processes to the total volume in L1 to L6 (Source Data 04, sheet 01). Values for L4-L6 are taken from Schmuhl-Giesen et al. 2022. **D,** Bar histogram showing the percentage (mean ± SD) of AZs covered by fine astrocytic processes, either on both sides, on one side or without any coverage, subdivided by their location on dendritic spines or shafts in L1 and L4 (Source Data 04, sheet 02). The horizontal bars in C and D indicate significant differences *$p < 0.05$; **$p < 0.01$.

Table 1: Comparative quantitative analysis of various structural and synaptic parameters in L1 of the human TLN.

	Layer	Mean ± SD	Median	IQR	CV	Variance	Skewness
		Synaptic boutons					
Surface area (µm²)	L1	5.48 ± 1.40	5.75	2.20	0.26	1.96	0.21
	L1a	4.58 ± 1.13	4.27	2.20	0.25	1.28	1.13
	L1b	6.39 ± 1.08	5.87	1.97	0.17	1.17	1.66
Volume (µm³)	L1	0.50 ± 0.19	0.38	0.33	0.39	0.04	0.13
	L1a	0.41 ± 0.21	0.49	0.41	0.50	0.04	0.64
	L1b	0.58 ± 0.17	0.48	0.31	0.30	0.03	1.71
		Active zones					
PreAZ surface area (µm²)	L1	0.20 ± 0.06	0.22	0.08	0.30	0.01	-1.53
	L1a	0.18 ± 0.08	0.22	0.14	0.43	0.00	-1.70
	L1b	0.22 ± 0.04	0.22	0.08	0.18	0.00	0.00
PSD surface area (µm²)	L1	0.22 ± 0.07	0.23	0.12	0.32	0.00	-0.91
	L1a	0.21 ± 0.10	0.23	0.19	0.47	0.01	-1.02
	L1b	0.22 ± 0.05	0.22	0.09	0.20	0.00	0.33
Cleft width (nm)	L1	L: 21.75 ± 2.72 C: 29.63 ± 2.36	L: 22.20 C: 29.73	L: 2.88 C: 3.19	L: 0.13 C: 0.08	L: 7.40 C: 5.56	L: -1.03 C: -0.96
	L1a	L: 20.17 ± 2.85 C: 29.06 ± 3.48	L: 21.44 C: 29.17	L: 5.25 C: 6.95	L: 0.14 C: 0.12	L: 8.10 C: 12.08	L: -1.61 C: -0.14
	L1b	L: 23.34 ± 1.70 C: 30.19 ± 0.92	L: 22,49 C: 30.19	L: 3.06 C: 1.84	L: 0.07 C: 0.03	L: 2,88 C: 0.85	L: 1.69 C: 0.00
		Mitochondria					
Volume (µm³)	L1	0.04 ± 0.02	0.04	0.03	0.35	0.00	-0.31
	L1a	0.04 ± 0.02	0.04	0.04	0.50	0.00	0.00
	L1b	0.05 ± 0.01	0.04	0.02	0.25	0.00	1.73
% to the total volume	L1	7.21 ± 1.10	7.56	1.81	0.15	1.21	-1.13
	L1a	7.18 ± 1.60	8.06	2.81	0.22	2.56	-1.73
	L1b	7.23 ± 0.68	7.17	1.35	0.09	0.46	0.42
		Synaptic vesicles					
Total number of SVs	L1	3430.97 ± 1773.77	3675.96	3386.86	0.52	3146276.00	-0.31
	L1a	2958.62 ± 1940.51	2980.76	3880.83	0.66	3765578.00	-0.05
	L1b	3903.32 ± 1852.23	4371.16	3614.75	0.47	3430758.00	-1.06
Volume (µm³)	L1	0.03 ± 0.02	0.03	0.03	0.60	0.00	0.94
	L1a	0.02 ± 0.01	0.02	0.02	0.50	0.00	0.00
	L1b	0.04 ± 0.02	0.04	0.04	0.50	0.00	0.00
Vesicle diameter (nm)	L1	25.03 ± 5.67	26.36	10.38	0.23	32.13	-0.31
	L1a	23.33 ± 5.27	25.88	9.56	0.23	27.74	-1.67
	L1b	26.73 ± 6.63	28.25	12.99	0.25	43.93	-0.98
	L1 *	30.29 ± 4.67	31.10	3.21	0.15	21.77	-2.60
	L1a*	28.11 ± 6.10	30.15	3.91	0.22	37.17	-2.04
	L1b*	31.88 ± 2.36	31.62	3.27	0.07	5.56	0.04
		Pool sizes of SVs					
Putative RRP p10 nm from the PreAZ	L1	3.60 ± 4.24	2.10	6.98	1.18	17.99	1.17
	L1a	5.90 ± 5.05	6.11	10.10	0.86	25.53	-0.18
	L1b	1.29 ± 1.87	0.41	3.41	1.44	3.49	1.65
Putative RRP p20 nm from the PreAZ	L1	19.05 ± 17.23	17.17	29.37	0.90	296.86	0.67
	L1a	25.04 ± 21.09	24.82	42.18	0.84	444.82	0.05
	L1b	13.07 ± 13.78	9.52	26.86	1.05	189.80	1.08
Putative RP 60-200 nm from the PreAZ	L1	463.00 ± 283.82	512.65	554.39	0.61	80553.57	-0.19
	L1a	390.12 ± 286.89	335.05	565.79	0.74	82304.11	0.83
	L1b	535.88 ± 321.16	690.25	584.03	0.60	103145.30	-1.66
Putative resting pool > 200 nm from the PreAZ	L1	2896.50 ± 1435.93	3120.39	2698.41	0.50	2061880.00	-0.38
	L1a	2515.02 ± 1588.99	2662.80	3167.65	0.63	2524880.00	-0.41
	L1b	3277.97 ± 1480.96	3577.98	2915.99	0.45	2193254.00	-0.87

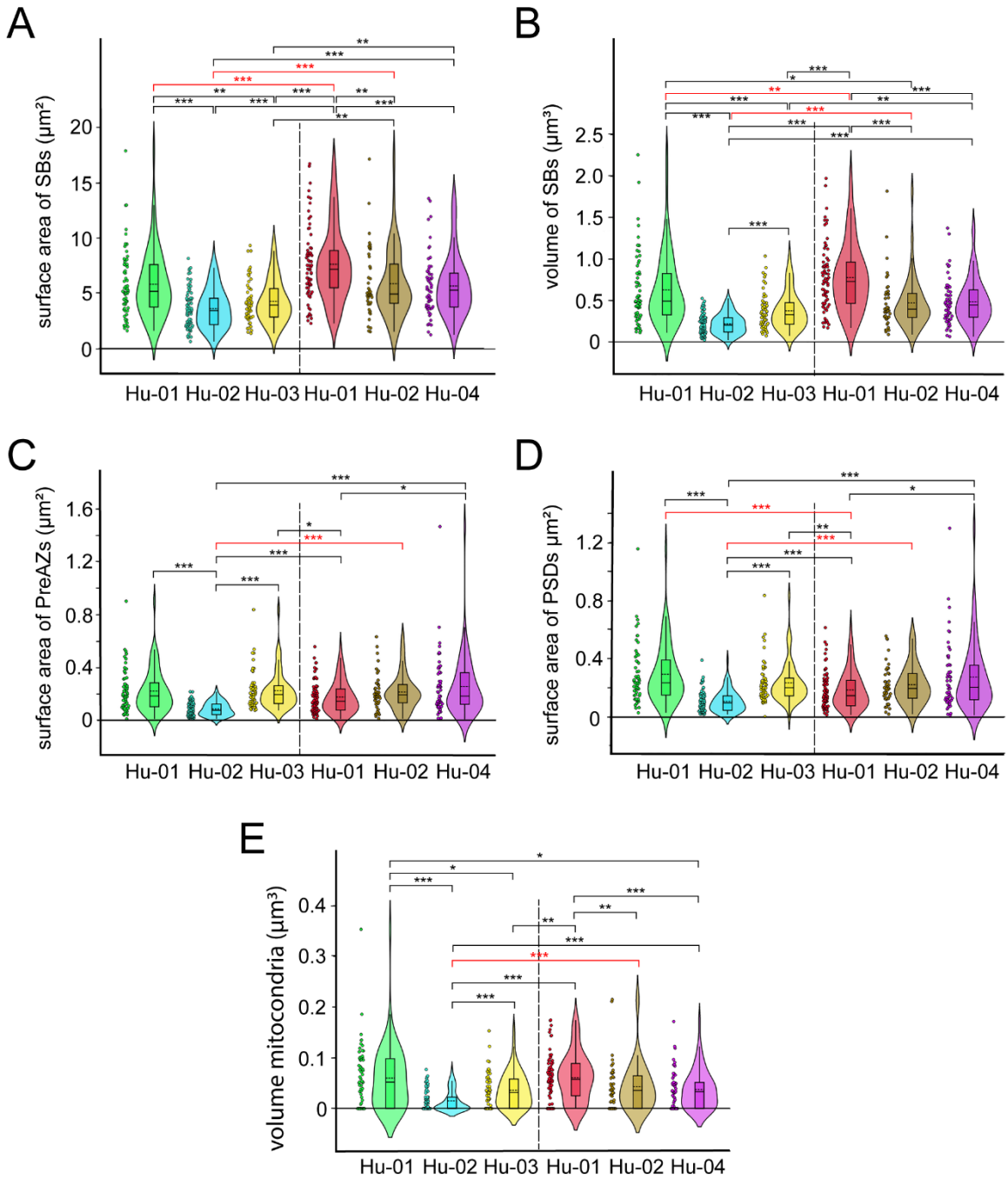
Summary of different structural parameter measurements (bold) from the detailed 3D-volume reconstructions of SBs in L1, separated for sublaminae. Values are presented as mean, SD, median, IQR, CV, skewness, and variance for each parameter for all patients studied. #: Values with skewness > 3 indicate a non-normal distribution. Abbreviations: IQR: Interquartile Range; CV: coefficient of variation; L: lateral; and C: central (Source Data 02; Source Data 03). *Values collected from TEM tomography.

1295 **Table 2: 'Docked SVs' in L1a and L1b of the human TLN.**

	Layer	Mean ± SD	Median	IQR	CV	Skewness	Variance
Number of docked vesicles	**L1** (n=360 SVs)	3.56 ± 1.36	4.00	3.00	0.38	-0.07	1.85
	L1a	3.71 ± 1.38	4.00	3.00	0.37	-0.16	1.89
	SBs on dendritic shafts (n=91 SVs)	3.64 ± 1.47	4.00	3.00	0.40	-0.08	2.16
	SBs on dendritic spines (n=98 SVs)	3.77 ± 1.31	4.00	2.00	0.35	-0.24	1.70
	L1b	3.42 ± 1.34	3.00	3.00	0.39	0.02	1.80
	SBs on dendritic shafts (n=87 SVs)	3.48 ± 1.45	3.00	3.00	0.42	0.04	2.09
	SBs on dendritic spines (n=84 SVs)	3.36 ± 1.25	3.00	2.50	0.37	-0.07	1.57

1296 Summary of the number of 'docked' SVs in L1, separately for both sublaminae as well as for the different target
1297 structures dendritic shafts vs. dendritic spines. Mean ± SD, Median, Interquartile Range (IQR), coefficient of variation
1298 (CV), Skewness, and Variance are given for each parameter in all patients studied (Source Data 03).

1299 **Supplemental Information**



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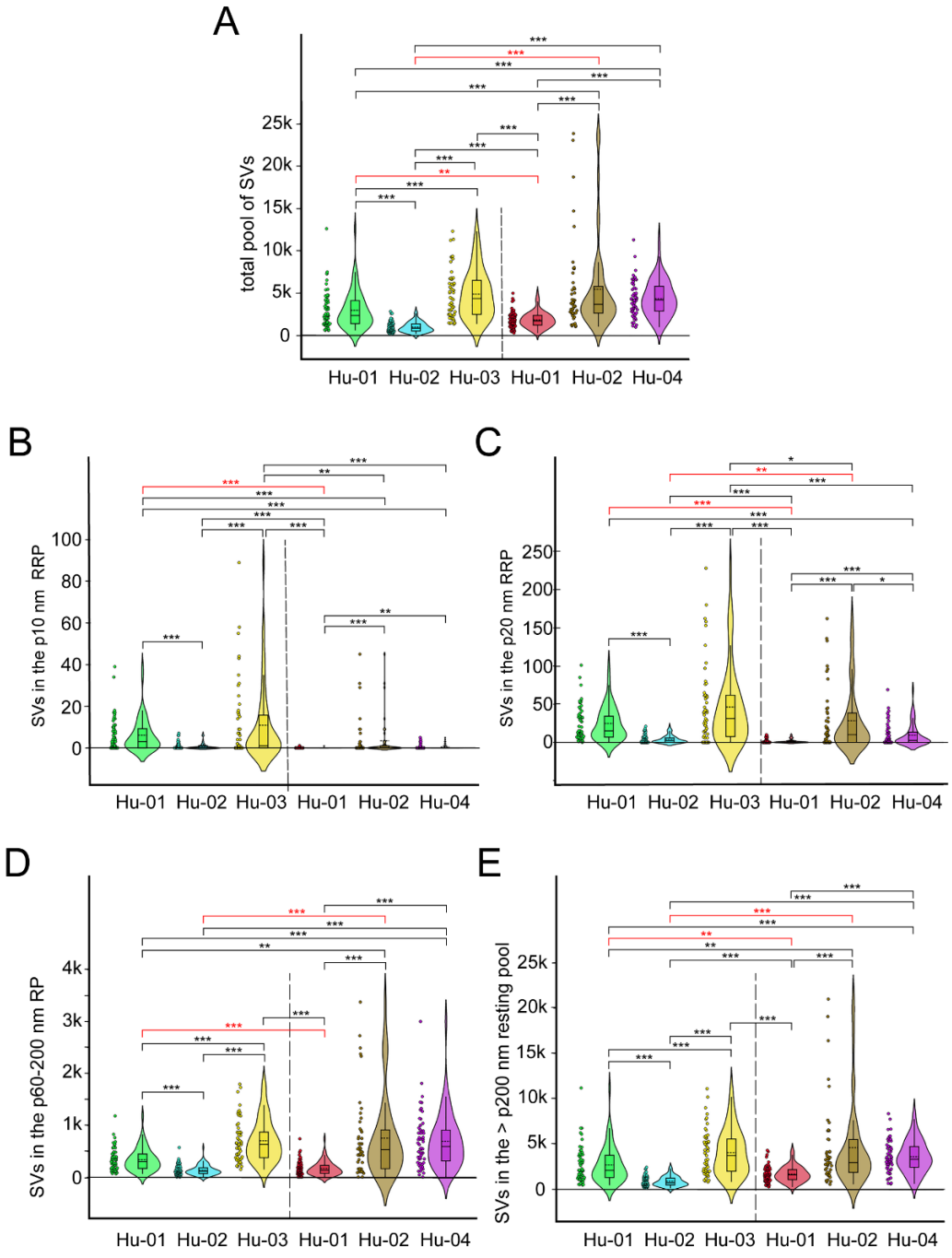
1301 **Supplemental Figure 1: Box plots of various structural parameters in L1 of the human TLN**
1302 Data distributions for each patient are indicated by the medians (horizontal bars), IQRs (framed areas),
1303 minimum and maximum (vertical lines) for the distribution of: A, Surface area of SBs; B, Volume of SBs;
1304 C, Surface area of PreAZs; D, Surface area of PSD; E, Volume of mitochondria. Significant differences
1305 between different patients are indicated by asterisks. Note that several structural parameters are significantly
1306 different. The significant bars highlighted in red indicate significant differences between sublaminae L1a
1307 and L1b in the same patient. *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$ (Source Data 02). Hu_01 and Hu_02
1308 were selected for both L1a and L1b in separate serial sections each.



Supplemental Figure 2: Box plots of various synaptic parameters in L1 of the human TLN
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,** Total pool of SVs; **B,** SVs in the p10 nm RRP; **C**, SVs in the p20 nm RRP; **D**, SVs in the p6-200 nm RP; **E**, SVs in the > p200 nm resting pool; Note that several structural parameters are not significantly different. The significant bars highlighted in red indicate significant differences between sublaminae L1a and L1b in the same patient. *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$ (Source Data 02). Hu_01 and Hu_02 were selected for both L1a and L1b in separate serial sections each.

1319 **Supplemental Table 1: Comparison of the synaptic density between L1a, L1b and L4 – L6 of the**
1320 **human TLN**

Cortical layers	L1a mean ± SD	L1b mean ± SD	Total L1 mean ± SD	L4 mean ± SD	L5 mean ± SD	L6 mean ± SD
Density of synaptic contacts/mm^3	$5.52*10^8$ ± $1.25*10^8$	$5.01*10^8$ ± $1.40*10^7$	$5.26*10^8$ ± $8.44*10^7$	$2.37*10^6$ ± $2.19*10^6$	$3.89*10^8$ ± $9.12*10^8$	$4.98*10^7$ ± $1.85*10^7$

1321 Values for L4–L6 are taken from: L4: Yakoubi et al. 2019b; L5: Yakoubi R., PhD Thesis 2023; L6:
1322 Schmuhl-Giesen et al. 2022.
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1327 **Supplemental Table 2: Patient's identity and medical background**

Patient's identity and medical background	Gender	Age (years)	Age at epilepsy onset (years)	Histo-Pathology	Antiepileptic drugs (pre-op)	Number of SBs recon-structed
Hu_01	♂	52	20	AHS	LEV, OXC, Perampanel	L1a: 63; L1b: 70
Hu_02	♀	27	16	Micro- and Astrogliosis	LEV, Topiramat, Zebinix	L1a: 72; L1b: 45
Hu_03	♀	65	1	Hippocampal sclerosis	LEV, LTG, Primidone	L1a: 55
Hu_04	♀	24	N/A	TLE	N/A	L1b: 56
Hu_05*	♀	36	4	GGL	LEV, LTG	
Hu_06*	♀	25	12	AHS	LTG	
Hu_07*	♀	25	23	GGL	Zebinix, LEV	
Hu_08*	♂	33	5	Gliosis	LEV, CBZ	
Hu_09*	♂	63	24	AHS	LEV, LTG, CBZ	

1328 Abbreviation: AHS: Ammon's horn sclerosis; CBZ: Carbamazepine; GGL: Ganglioglioma; LEV:
1329 Levetiracetam; LTG: Lamotrigine; OXC: Oxcarbazepin; TLE: Temporal Lope Epilepsy. *Hu_05 – Hu_09
1330 were only used to analyze the astrocytic coverage of AZs in L4.