

Cellular organization of cortical barrel columns is whisker-specific

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The cellular organization of the cortex is of fundamental importance for elucidating the structural principles that underlie its functions. It has been suggested that reconstructing the structure and synaptic wiring of the elementary functional building block of mammalian cortices, the cortical column, might suffice to reverse engineer and simulate the functions of entire cortices. In the vibrissal area of rodent somatosensory cortex, whisker-related “barrel” columns have been referred to as potential cytoarchitectonic equivalents of functional cortical columns. Here, we investigated the structural stereotypy of cortical barrel columns by measuring the 3D neuronal composition of the entire vibrissal area in rat somatosensory cortex and thalamus. We found that the number of neurons per cortical barrel column and thalamic “barreloid” varied substantially within individual animals, increasing by ~2.5-fold from dorsal to ventral whiskers. As a result, the ratio between whisker-specific thalamic and cortical neurons was remarkably constant. Thus, we hypothesize that the cellular architecture of sensory cortices reflects the degree of similarity in sensory input and not columnar and/or cortical uniformity principles.

soma counts | NeuN | GAD67 | VPM | barrel cortex

Two major concepts of cortical neuronal organization have been proposed. Structurally, correlations between stereology-based measurements (1) of neuron density and cortical thickness resulted in the hypothesis of structural uniformity, arguing that the number of neurons beneath a square millimeter of cortical surface is constant and independent of cortical area and species (2, 3). Functionally, cortex is organized in a columnar fashion, reflecting similar neuronal activity along the vertical cortex axis in response to peripheral stimuli (4–8). Similar spatial extents of functional cortical columns in the horizontal plane, combined with the idea of cortical uniformity, resulted in the notion that a stereotypic columnar network may also represent the elementary structural building block of sensory cortices (9). In combination, the two concepts thus suggested a common organization of all sensory cortices, which led to reverse engineering and simulation efforts that build up large-scale network models of repeatedly occurring identical cortical circuits (10, 11).

The ideal model system for investigating columnar structure and function is the vibrissal area of rodent somatosensory cortex. There, “barrels” of neurons in layer 4 (L4) have been identified as somatotopically organized structural correlates of peripheral receptor organs (i.e., facial whiskers). Whisker/barrel columns have thus been regarded as both structural and functional elementary cortical units (12–14). To investigate the structural stereotypy of cortical barrel columns, independent of the drawbacks associated with stereology (i.e., extrapolations from small sampling regions), we decided to locate each excitatory and inhibitory neuron soma within the entire volume of interest. Using high-resolution, large-scale confocal microscopy (15) and automated image-processing routines (16), we found that the number of neurons per barrel column increased by ~2.5-fold from columns that correspond to the dorsal facial whiskers (A-row) to columns corresponding to the ventral whiskers (E-row). Moreover,

cortical thickness increased by ~500 μm from A- to E-rows, resulting in whisker-specific laminar neuron profiles, layer locations, and thicknesses. Further, the distributions of excitatory and inhibitory neurons outside the L4 barrels were indistinguishable between barrel columns, the septa (the cortex separating the barrel columns) (14) and the dysgranular zones (DZ) surrounding the vibrissal cortex (17).

We performed the same analyses for the ventral posterior medial division (VPM) of rat thalamus, which provides whisker-specific input to the vibrissal cortex (18–20). Again, we found that the number of neurons per whisker (i.e., within so-called “barreloids”) (21) was constant within a whisker row, but increased by ~2.5-fold from the A- to the E-row. Consequently, the ratio between neurons per barrel (column) and respective barreloid was remarkably constant. This whisker-specific cellular organization is in contrast to the ideas of columnar and cortical uniformity, questioning the stereology-based concept that mammalian cortices are composed of stereotypical elementary building blocks.

Results

Across-Animal Variability in Rat Vibrissal Cortex. We measured the number of excitatory and inhibitory neurons within 24 barrel columns (α - δ , A1–E4) and the septa between them in four different rats (Fig. 1 A, C, and D; *SI Appendix, Table S1*). The average total number of neurons in this large portion of the vibrissal cortex was $529,715 \pm 39,104$ (mean \pm SD). A fraction of 87% of the neurons in the vibrissal cortex were excitatory and 13% inhibitory. Extrapolating L4 barrel boundaries toward the pia and white matter (WM), 81% of all neurons were located within barrel columns and 19% in the septa between them. The total volume of this part of the vibrissal cortex after perfusion and fixation (22) was $6.60 \pm 0.58 \text{ mm}^3$, which is consistent with

Significance

Cortical columns are thought to be the elementary functional building blocks of sensory cortices. Here we show that the cellular architecture of cortical “barrel” columns in rodent somatosensory cortex is not stereotypic, but specific for each whisker on the animals’ snout. Our findings challenge the concepts underlying contemporary simulation efforts that build up large-scale network models of repeatedly occurring identical cortical circuits.

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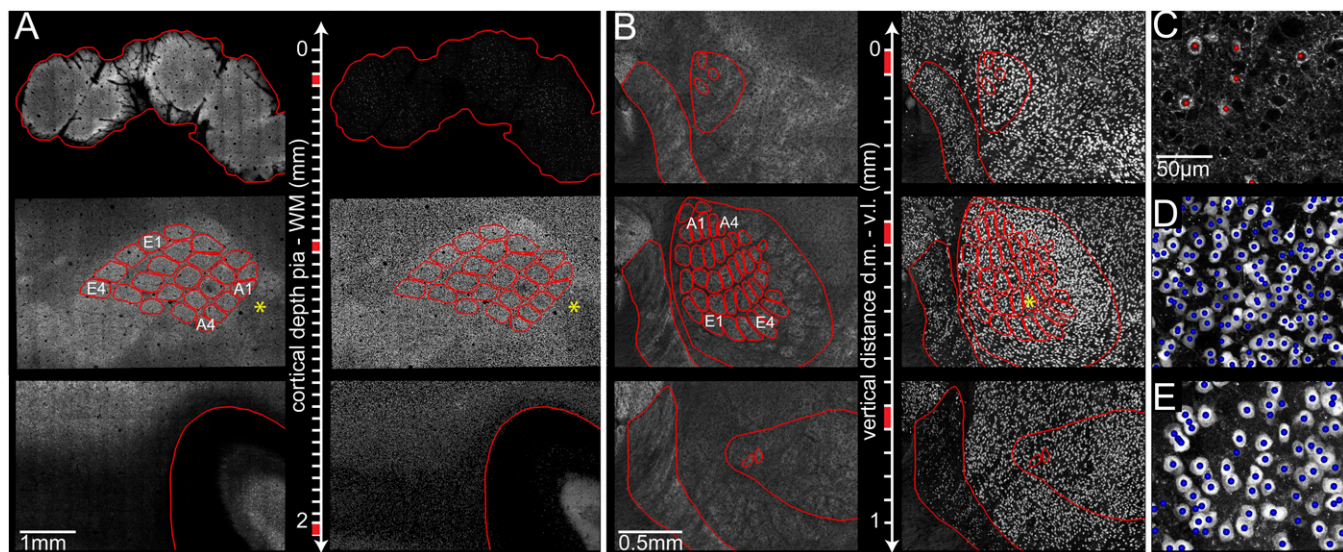


Fig. 1. Automated detection of all excitatory and inhibitory neuron somata in the vibrissal areas of rat somatosensory cortex and thalamus. (A) Large-scale, high-resolution confocal image stacks from 50- μm -thick brain sections, cut tangentially to the cortical surface from the pia to the WM. (Left) GAD67 projection images allow delineation of anatomical reference structures (red) in each section, such as pia, L4 barrels, and WM. (Right) NeuN projection images in the same sections. (B) Confocal image stacks from 50- μm -thick sections of vibrissal thalamus, cut tangentially from the dorsal medial (d.m.) to the ventral lateral (v.l.) direction. (Left) GAD67 projection images allow delineation of anatomical reference structures (red), such as RT (Left), VPM (Right), and individual barreloids. (Right) NeuN projection images in the same sections. (C) Optical section of GAD67 image stack from A (*) superimposed with landmarks representing automatically detected inhibitory somata (red). (D) NeuN-positive somata were automatically detected within the same area. (E) Area from B (*) with automatically detected NeuN-positive neuron somata. Brightness has been adjusted in all panels for visualization purposes.

previous measurements of the cortex geometry using cytochrome oxidase as a marker to reveal the barrels ($6.53 \pm 0.75 \text{ mm}^3$) (23). The across-animal variabilities in total neuron numbers (SD of

mean: 7.4%) and volume (8.8%) were similar. Consequently, the average neuron density across the entire vibrissal cortex was remarkably preserved ($80,419 \pm 3,688 \text{ mm}^{-3}$); the same was true

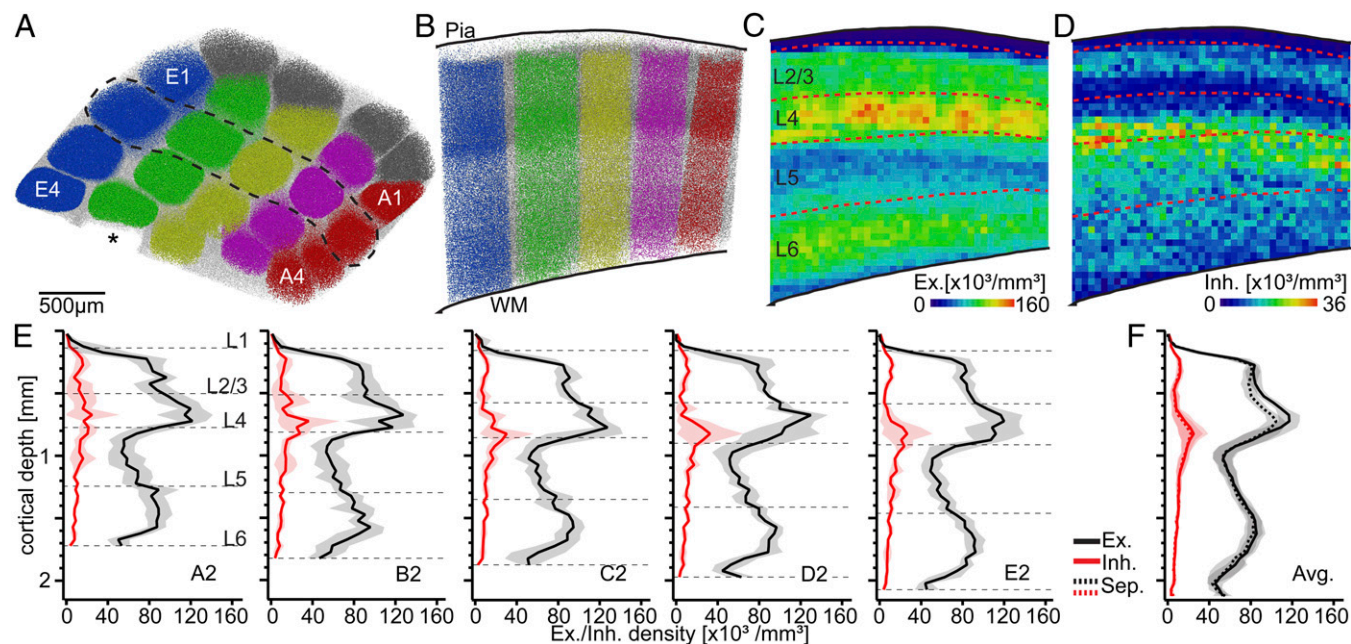


Fig. 2. Whisker-specific laminar cellular organization of rat vibrissal cortex. (A) Tangential view of all neuron somata in the vibrissal cortex of one animal. Somata are assigned to their closest barrel column [row colors: A (red), B (pink), C (yellow), D (green), E (blue), Greek arc (gray)] or to the septum (white). *D5 barrel column excluded from analysis. (B) Semicoronal view of the somata within the dashed region in A. A 3D reconstruction of pia and WM surfaces allows for determining the position of all neuron somata with respect to cytoarchitectonic layer borders (20). (C) A 2D average projection of the 3D excitatory neuron density. L4 barrels are clearly visible as segregated spots of high neuron density (24). (D) A 2D average projection of the 3D inhibitory neuron density. Segregation between barrels and septa is not evident. L2 and upper L5 are separable as bands of high inhibitory neuron density, as reported previously (22). (E) Average distribution of excitatory and inhibitory neuron somata along the vertical column axis for columns in arc-2. Shaded regions are ± 1 SD. Dashed lines represent column-specific layer borders. (F) Average distribution of excitatory/inhibitory somata across all barrel columns and septa.

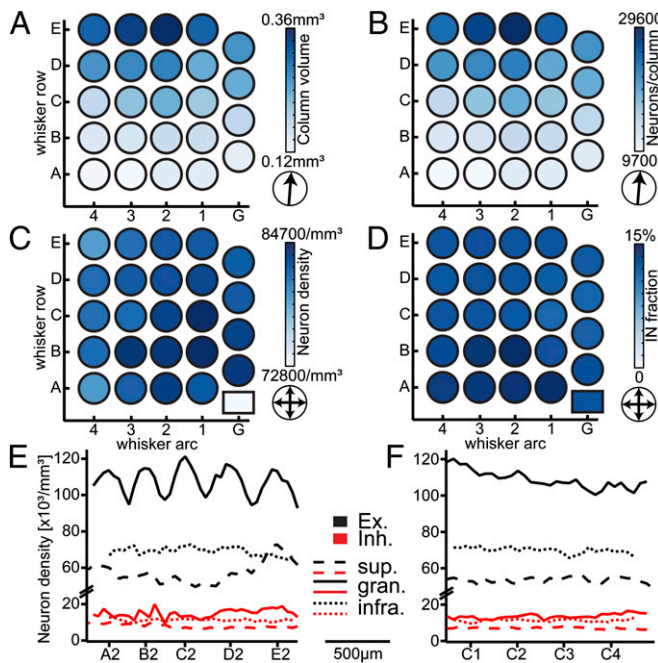


Fig. 3. Whisker-specific horizontal cellular organization of rat vibrissal cortex. (A) Average volume per barrel column based on the four vibrissal cortices analyzed, showing a significant increase from the A- to the E-row. (B) Average number of all neurons (excitatory and inhibitory) per barrel column, increasing from the A- to the E-row concomitantly with the column volume. (C) The average neuron density (excitatory and inhibitory) per barrel column is constant across the barrel field and larger than septal neuron density (box). (D) The average fraction of inhibitory neurons (IN) is similar across the barrel field and does not differ between columns and the septum (box). (E) The average distribution of excitatory/inhibitory neurons in different cortical layers, measured along arc-2 (from left to right: A2–E2), shows a clear separation into barrel columns and septa only in L4, where excitatory neurons delineate the barrels. (F) The average distribution of excitatory/inhibitory neurons in different cortical layers, measured along the C-row (from left to right: C1–C4), is not indicative of a separation between barrel columns and septa at the cellular level.

for the average density in columnar ($82,402 \pm 4,011 \text{ mm}^{-3}$) and septal ($72,792 \pm 2,419 \text{ mm}^{-3}$) regions, respectively. There was no difference between the neuron density in the septa between whisker rows ($67,078 \pm 4,751 \text{ mm}^{-3}$) and in the DZ surrounding the vibrissal cortex ($68,236 \pm 2,226$ anterior medial to the E-row and $66,311 \pm 1,084 \text{ mm}^{-3}$ posterior lateral to the A-row; *SI Appendix, Fig. S1*).

Across-Animal Variability in Rat Vibrissal Thalamus. Labeling brain sections with GAD67 did not only reveal the columnar organization of rat vibrissal cortex, as previously reported (24), but also showed the segregation of VPM thalamus into barreloids (Fig. 1B). Using the same methodology as for the vibrissal cortex, we measured the number of excitatory and inhibitory neurons for the respective 24 barreloids in three different rats (Fig. 1E). The average total number of neurons in this portion of vibrissal thalamus was $9,963 \pm 718$. As for the vibrissal cortex, the variability in neuron numbers across animals was small (7.2%). All neurons in VPM thalamus were excitatory (i.e., GAD67 negative). The total volume of this part of vibrissal thalamus (i.e., convex hull around 24 barreloids; *Materials and Methods*) was $0.19 \pm 0.03 \text{ mm}^3$, resulting in an average neuron density across the entire VPM of $52,494 \pm 5,082 \text{ mm}^{-3}$. Densities in VPM within and above/below barreloids ($51,507 \pm 4,422$ and $54,440 \pm 6,559 \text{ mm}^{-3}$, respectively) were larger compared with the surrounding thalamic nuclei, with $49,680 \pm 1,097$ and $41,477 \pm 3,612$

mm^{-3} in nucleus reticularis (RT) and posterior medial nucleus (POM), respectively.

Whisker-Specific Laminar Organization. Each detected soma was assigned to its nearest barrel column or to the septum, respectively (Fig. 2A and B). The resultant column/septum-specific 3D distribution of excitatory (Fig. 2C) and inhibitory somata (Fig. 2D) could thus be analyzed with respect to previously defined cytoarchitectonic layers (20). Cortical thickness (i.e., pia–WM distance along the respective vertical column axis) (23) increased substantially across the vibrissal cortex, being thinnest at the barrel column corresponding to the α -whisker ($1,612 \pm 36 \mu\text{m}$) and thickest at the E3 whisker representation ($2,089 \pm 15 \mu\text{m}$). The increase in cortical thickness was linear across whisker rows (linear regression: $R^2 = 0.99$, $P = 0.001$). Consequently, the density distributions of individual barrel columns were “stretched” along their respective vertical axis, resulting in column-specific depth locations and thicknesses of the respective cytoarchitectonic layers (Fig. 2E, dashed lines). In contrast, the height of the L4 barrel increased sublinearly ($R^2 = 0.9$, $P = 0.10$). Thus, the depth of granular L4 was more constant across the vibrissal cortex than the respective cortical thickness. For example, cortical thickness increased from the A2 ($1,760 \pm 39 \mu\text{m}$) to the E2 column ($2,063 \pm 50 \mu\text{m}$) by $303 \mu\text{m}$, and the depth of the L4 peak (Gaussian approximation) shifted by $116 \mu\text{m}$ (from 647 to $763 \mu\text{m}$ below the pia surface), whereas the L5/6 peak shifted $\sim 2.4\times$ more, i.e., by $275 \mu\text{m}$ (from $1,409$ to $1,684 \mu\text{m}$).

The density of excitatory and inhibitory neurons within each of the respective layers was remarkably constant across the vibrissal cortex. The average neuron density was $61,603 \pm 3,721 \text{ mm}^{-3}$ (SD of mean: 6.0%) within supragranular (s) L1–3, $122,931 \pm 6,204 \text{ mm}^{-3}$ (5.1%) within granular (g) L4 and $79,092 \pm 5,383 \text{ mm}^{-3}$ (6.8%) within infragranular (i) L5–6. The same was true for the average density in columnar (s: $63,878 \pm 4,329$; g: $126,145 \pm 6,298$; i: $79,424 \pm 5,441 \text{ mm}^{-3}$) and septal (s: $56,061 \pm 2,301$; g: $111,293 \pm 7,558$; i: $77,090 \pm 5,040 \text{ mm}^{-3}$) regions, respectively. Because neuron densities within a layer were constant, but layer depths and thicknesses changed across the vibrissal cortex, the numbers of excitatory and inhibitory neurons within cytoarchitectonic layers were highly whisker-specific (*SI Appendix, Tables S2–S6*).

The relative proportions of neurons per layer were, however, virtually identical for all barrel columns. A fraction of $24 \pm 1\%$ of all neurons within a barrel column were located within supragranular layers (L1: $0.5 \pm 0.1\%$; L2/3: $23.3 \pm 1.1\%$), $25.2 \pm 0.0\%$ in granular L4 and $51 \pm 2\%$ in infragranular layers (L5: $24.2 \pm 0.9\%$; L6: $26.8 \pm 1.2\%$). The proportion of neurons within the L4 barrel was independent of the respective cortical thickness (Pearson’s correlation coefficient: $r = 0.02$, two-tailed t test: $P = 0.93$) and significantly correlated with (i.e., predicted) the respective supragranular ($r = -0.46$, $P = 0.02$) and infragranular ($r = -0.77$, $P < 10^{-3}$) proportions. In contrast, supra- and infragranular proportions were uncorrelated ($r = 0.24$, $P = 0.26$). Consequently, the largely preserved vertical extent and depth location of the L4 barrels caused the constant laminar neuronal composition of cortical barrel columns, despite substantial whisker-specific increases in neuron numbers and cortical thickness.

Whisker-Specific Horizontal Organization. We calculated the direction of the gradient within the horizontal plane of the vibrissal cortex (Fig. 3, arrows) for the volume, neuron density, neuron number, and fraction of inhibitory neurons per barrel column, respectively. The horizontal gradient in column volume revealed a “rowish” organization of the vibrissal cortex (23). Barrel columns within the same whisker row displayed almost identical volumes (Table 1), whereas the column volume increased in an orderly manner from the A- toward the E-row by a factor of ~ 2.5 (Fig. 3A; $P < 10^{-15}$, one-way ANOVA). In contrast, average neuron densities did not differ between columns, as indicated by the absence of a horizontal gradient across the vibrissal cortex (Fig. 3C; $P = 0.49$, two-tailed t test). Consequently, the average

Table 1. Whisker-specific organization in rat vibrissal cortex (S1) and thalamus (VPM)

Whisker	Volume S1	Neurons S1	Excitatory S1	Inhibitory S1	Volume VPM	Neurons VPM	Ratio S1/VPM
α	0.14 ± 0.03	11,781 ± 2,341	10,244 ± 2,235	1,536 ± 298	0.35 ± 0.05	186 ± 30	63
β	0.18 ± 0.02	15,002 ± 679	13,161 ± 857	1,841 ± 640	0.42 ± 0.06	199 ± 23	75
γ	0.24 ± 0.04	19,663 ± 1,857	17,270 ± 1,406	2,393 ± 857	0.56 ± 0.07	282 ± 11	70
δ	0.27 ± 0.03	21,919 ± 1,257	19,155 ± 1,363	2,764 ± 849	0.49 ± 0.06	246 ± 22	89*
A1	0.14 ± 0.02	11,832 ± 2,100	9,346 ± 1,480	1,675 ± 486	0.35 ± 0.05	196 ± 23	60
A2	0.14 ± 0.03	11,978 ± 2,415	9,424 ± 1,789	1,624 ± 370	0.31 ± 0.05	198 ± 28	61
A3	0.12 ± 0.01	9,741 ± 941	8,066 ± 990	1,351 ± 160	0.21 ± 0.04	132 ± 16	74
A4	0.12 ± 0.03	9,660 ± 2,190	8,228 ± 1,679	1,432 ± 655	0.14 ± 0.04	89 ± 20	108*
A-row	0.13 ± 0.01	10,803 ± 1,275	8,766 ± 719	1,521 ± 154	0.25 ± 0.09	154 ± 53	65 ± 8
B1	0.17 ± 0.03	14,387 ± 1,534	12,520 ± 1,806	1,866 ± 402	0.54 ± 0.06	295 ± 40	49
B2	0.17 ± 0.03	14,547 ± 1,888	11,953 ± 2,279	2,089 ± 392	0.54 ± 0.10	306 ± 26	47
B3	0.15 ± 0.03	12,999 ± 2,493	10,292 ± 1,951	1,741 ± 346	0.40 ± 0.09	227 ± 35	57
B4	0.15 ± 0.02	12,393 ± 1,949	10,726 ± 1,952	1,667 ± 242	0.26 ± 0.03	149 ± 8	83
B-row	0.16 ± 0.01	13,581 ± 1,054	11,373 ± 1,039	1,841 ± 185	0.43 ± 0.13	244 ± 73	59 ± 17
C1	0.21 ± 0.04	17,491 ± 2,406	15,459 ± 2,538	2,032 ± 551	0.64 ± 0.06	329 ± 12	53
C2	0.24 ± 0.02	19,707 ± 1,174	17,195 ± 1,545	2,511 ± 661	0.65 ± 0.06	350 ± 3	56
C3	0.22 ± 0.03	17,531 ± 1,496	15,273 ± 1,661	2,258 ± 435	0.47 ± 0.05	253 ± 19	69
C4	0.18 ± 0.03	14,918 ± 3,154	13,028 ± 3,075	1,890 ± 278	0.38 ± 0.03	218 ± 29	69
C-row	0.21 ± 0.02	17,412 ± 1,959	15,239 ± 1,709	2,173 ± 272	0.54 ± 0.13	287 ± 62	62 ± 8
D1	0.24 ± 0.01	20,078 ± 1,852	17,588 ± 2,191	2,489 ± 895	0.60 ± 0.06	286 ± 4	70
D2	0.28 ± 0.02	23,382 ± 1,484	20,377 ± 2,124	3,005 ± 844	0.64 ± 0.06	311 ± 7	75
D3	0.28 ± 0.04	22,696 ± 3,007	19,776 ± 3,304	2,920 ± 535	0.61 ± 0.12	296 ± 37	77
D4	0.26 ± 0.01	21,619 ± 760	18,862 ± 1,443	2,758 ± 686	0.53 ± 0.08	270 ± 26	80
D-row	0.27 ± 0.02	21,944 ± 1,440	19,151 ± 1,214	2,793 ± 227	0.60 ± 0.05	291 ± 17	76 ± 4
E1	0.31 ± 0.04	25,459 ± 1,879	22,183 ± 2,412	3,276 ± 836	0.65 ± 0.08	298 ± 55	85
E2	0.36 ± 0.06	29,563 ± 3,604	25,813 ± 4,133	3,750 ± 897	0.77 ± 0.15	346 ± 41	85
E3	0.34 ± 0.05	27,965 ± 4,344	24,391 ± 4,890	3,575 ± 698	0.86 ± 0.07	403 ± 33	69
E4	0.31 ± 0.05	24,671 ± 3,743	21,587 ± 4,118	3,085 ± 395	0.78 ± 0.06	361 ± 41	68
E-row	0.33 ± 0.02	26,914 ± 2,256	23,493 ± 1,961	3,421 ± 298	0.76 ± 0.08	352 ± 43	77 ± 10
Mean	0.22 ± 0.07	17,958 ± 5,800	15,497 ± 5,266	2,314 ± 697	0.51 ± 0.18	259 ± 78	68 ± 11

All numbers are mean ± SD (S1: $n = 4$; VPM: $n = 3$). Volumes in S1 and VPM are given in cubic millimeters and 10^{-2} mm^3 , respectively.

*Barreloid Delta and A4 were excluded from the ratio analysis due to difficulties to identify their boundaries.

number of neurons per cortical barrel column followed the gradient in column volume, resulting in relatively constant neuron numbers for columns within the same whisker row (Table 1) and an orderly ~ 2.5 -fold increase from the A- ($10,803 \pm 1,275$ neurons) toward the E-row ($26,914 \pm 2,256$ neurons) (Fig. 3B; $P < 10^{-15}$, one-way ANOVA).

Fig. 3C (lower right rectangle refers to the septum) indicates a significant drop in neuron density between barrel columns and septa. In contrast, the relative proportion of inhibitory neurons was independent of barrel column identity or septal location (Fig. 3D; $P = 0.65$, two-tailed t test). Moreover, the average 1D profiles in Fig. 2F revealed that the density of inhibitory neurons was nearly identical between columns ($10,826 \text{ mm}^{-3}$) and septa ($9,516 \text{ mm}^{-3}$). The distribution of excitatory neurons differed between columns and septa, but differences were limited to L4. There, the density of excitatory cells dropped from barrels to the septum by up to 17%, reflecting a decrease from $\sim 120,000$ to $\sim 100,000$ neurons per cubic millimeter. Fig. 3E and F further illustrates this finding for the distribution of excitatory and inhibitory neurons within supragranular, granular, and infragranular layers, respectively. A significant separation between columns and septa was only evident for the distribution of excitatory neurons within granular L4 along the whisker arc (i.e., between whisker rows). Consequently, outside the L4 barrel, the cellular organization of excitatory and inhibitory neurons was virtually identical in barrel columns, septa, and the DZ surrounding the vibrissal cortex.

Organization Between Vibrissal Thalamus and Cortex. We also determined the neuron numbers, volumes, and densities of each individual VPM barreloid (Table 1 and Fig. 4A–C). The relationship between barreloid volume and neuron density differed from the vibrissal cortex. The neuron density was not constant

across VPM, but increased from the E1 test toward the A4 barreloid by $\sim 50\%$. Conversely, barreloid volume increased in the opposite direction from the A4 toward the E3 barreloid. Consequently, the number of neurons per barreloid was, similar to barrel columns, remarkably constant within a whisker row, increasing in an orderly manner by ~ 2.5 -fold from the A- (154 ± 53 neurons) toward the E-row (352 ± 43 neurons; $P < 10^{-10}$). Thus, the ratio between the number of neurons per barrel column and the number of neurons within the respective barreloid was relatively constant (i.e., 68 ± 11). Because the depth locations and heights of the L4 barrels were more preserved than cortical thickness, we calculated the ratios between barrel columns and barreloids for supragranular, granular, and infragranular layers individually (Fig. 4D–F). We found that the correlation between neuron numbers in the barreloid and the L4 barrel ($R^2 = 0.76$) exceeded the ones with the other layers ($R^2 = 0.68$ for supragranular and $R^2 = 0.62$ for infragranular layers), reflecting a remarkably constant neuron ratio of 18 ± 3 between each barrel and the respective barreloid (i.e., no significant gradient in neuron ratios, $P = 0.14$; two-tailed t test).

Discussion

Previous Studies of Cortical Cellular Organization. Several studies have investigated the cellular organization of sensory cortices, including rat vibrissal cortex (2, 3, 22, 24–26). Most of these studies were based on stereology, measuring local neuron densities and extrapolating them to larger reference volumes. Our results indicate that such approaches are problematic. First, neuron densities are not uniform, resulting in whisker-specific profiles along the vertical axis. Second, the sizes of the reference volumes (i.e., barrel columns) vary substantially across the vibrissal cortex, resulting in row-specific distributions within the

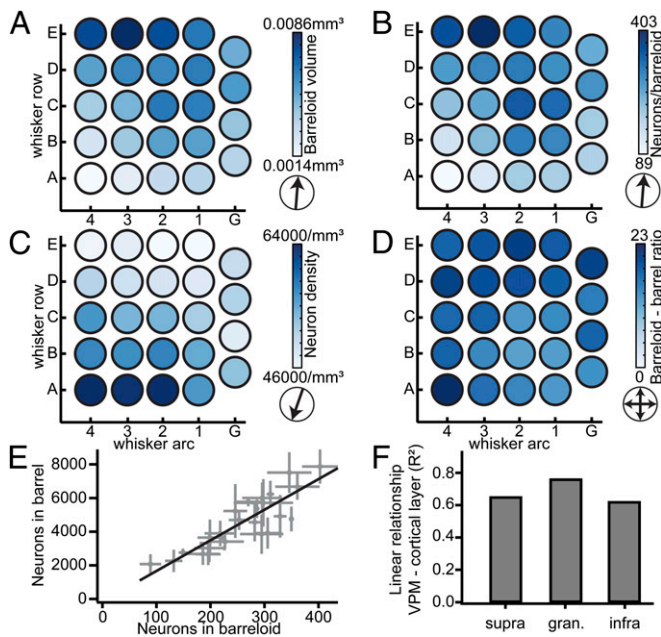


Fig. 4. The ratio between whisker-specific cortical and thalamic neurons is constant. (A) The average volume per barreloid across VPM increases from the A- to the E-row, as in vibrissal cortex. (B) The average number of neurons per barreloid also increases from the A- to the E-row. (C) The average neuron density per barreloid increases from the E- toward the A-row. (D) The average ratio between the number of neurons per barrel and corresponding barreloid is highly preserved. (E) Relationship between the number of neurons per barreloid in VPM and the number of neurons in the corresponding barrel is linear. Error bars are ± 1 SD. (F) The linear relationship between neurons per barreloid and the respective number of target neurons in cortex is more pronounced for granular L4, compared with supra- and infragranular layers.

horizontal plane. Consequently, measuring the detailed 3D geometry of the entire vibrissal cortex, and detecting all neuron somata with respect to this anatomical reference frame, indicates that the variability across animals in neuron numbers and 3D distributions is much smaller compared with previous studies (e.g., ranging from neuron densities of 48,000 to 77,000 per cubic millimeter (25, 26)). Our automated counts further confirm previous manual (22, 24) and automated (27) counts of NeuN- and GAD67-positive neurons obtained for smaller datasets. Consequently, because neither the definition of a reference volume, nor the assumption of homogeneous neuron distributions within these volumes is required, we argue that our approach can be regarded as a robust alternative to stereology for revealing the cellular organization principles within any brain area of interest.

Cellular Organization Beyond Structural Uniformity. Stereology-based studies gave rise to idea of structural uniformity, suggesting that the number of neurons beneath the cortical surface is constant and independent of cortical area and species. The validity of this principle depends on whether differences in cortical thickness are compensated by changes in neuron densities. Measuring all these quantities directly, we showed that this hypothesis fails (*SI Appendix, Fig. S2*), at least for rat vibrissal cortex, which has been an integral part of the original studies that suggested structural uniformity (2, 3). Nevertheless, the structural uniformity principle gave rise to concepts about cortical organization in general. Though it is largely undisputed that cortex is subdivided into functional columns of various kinds (for a review, see ref. 28), the existence of anatomical counterparts remains controversial. Due to their unique anatomical segregation in L4, cortical barrel columns are usually regarded as potential candidates for such an anatomical equivalent. In combination with

the structural uniformity principle, it was even suggested that understanding the structural and functional principles of one average cortical barrel column will serve as a template for understanding all cortical areas (9). This is arguably not the case. Our results show that neither does structural uniformity apply to rat vibrissal cortex, nor is the cellular organization of barrel columns identical within the same animal.

As an alternative organizational principle to columnar and cortical uniformity, we put forward the following hypothesis: The cellular organization of cortical barrel columns is primarily shaped by the similarity of sensory inputs from whiskers within the same row. For example, the large whiskers (arc 1–4) located in the E-row are closer to the ground and may touch it more frequently and/or are better suited to discriminate between textures than the corresponding whiskers within the A-row. In support of this idea are studies showing that sensory receptor densities around individual whiskers are proportional to the size of the corresponding “barrelettes” in brainstem (29), as well as to the number of neurons in the corresponding L4 barrel (30).

Despite substantial whisker-specific differences in total neuron numbers, some important aspects of the cellular composition of cortical barrel columns are highly preserved, e.g., the relative laminar neuronal composition, the fraction of inhibitory neurons, and, most importantly, the neuron ratio between the barrel and the respective barreloid. It remains to be investigated whether these preserved quantities are indicative of common elementary circuits and functions shared by all whiskers (e.g., for object localization), whereas progressively increasing neuron numbers may reflect additional functionalities and/or higher sensitivities depending on the whisker position with respect to the ground (e.g., for texture discrimination).

Conclusion

Using quantitative methods to reconstruct the cellular architecture of the entire rat vibrissal cortex and thalamus, we showed that cortical barrel columns and thalamic barreloids are organized in a whisker-specific manner. Our results are in line with previous hypotheses suggesting that information from the sensory sheet guides the features of cortical maps and that cortex is not constrained to form stereotypic columnar units (31). In case of rat vibrissal cortex, similar sensory input from whiskers within the same row may be manifested by adding a whisker-specific number of neurons to L4 during development (as reviewed in ref. 32; a process that is critically dependent on and guided by VPM input) (33), on top of a cellular architecture that is otherwise indistinguishable from the surrounding dysgranular cortices. Consequently, our findings argue against the views that cortical barrel columns represent stereotypic anatomical correlates of functional columns and that cortical organization follows a structural uniformity rule. It remains thus to be shown whether large-scale network models, based upon repeatedly occurring identical cortical columns (10), will elucidate general mechanisms that underlie cortical organization and sensory information processing.

Materials and Methods

Sample Preparation. Experimental procedures were conducted in accordance with the German Animal Welfare Act and approved by the Institutional Animal Care and Use Committee of the Max Planck Florida Institute for Neuroscience. Histology was conducted as reported previously (24). Briefly, Wistar rats (aged 28–29 d) were perfused transcardially, and brains were removed and fixed with paraformaldehyde. For neuron counting in cortex, 43–48 consecutive Vibratome sections of 50 μ m thickness were cut tangentially to vibrissal cortex, and for neuron counting in thalamus, 15–18 sections 50 μ m thick were cut semicorally, i.e., approximately tangential to the barreloid field (34). Sections were double-immunolabeled for GAD67 (35–37) and NeuN (38).

Image Acquisition. Images were acquired using a prototype confocal laser scanning system (based on Leica Application Suite Advanced Fluorescence SP5; Leica Microsystems) equipped with a glycerol/oil immersion objective (HC PL APO 20 \times , 0.7 N.A.), a tandem scanning system (Resonance Scanner),

spectral detectors with hybrid technology (GaAsP photocathode), and mosaic scanning software [Matrix Screener (beta version), provided by Frank Sieckmann, Leica Microsystems]. Mosaic image stacks of volumes up to $5 \times 3.5 \times 0.05$ mm (in cortex) and $3 \times 3 \times 0.05$ mm (in thalamus) were acquired at a resolution of $0.36075 \times 0.36075 \times 0.5$ μ m per voxel (2.5 \times digital zoom, 8 \times line average, 8-kHz scanning speed, $\sim 15 \times 10$ and $\sim 9 \times 9$ fields of view in cortex and thalamus, respectively) for each consecutive brain section.

Image Processing. NeuN-positive somata were detected in each confocal image stack using a previously described automated counting algorithm (16). Each detected soma is represented by a 3D position landmark. The accuracy and robustness of the algorithms has been validated against manual counts performed by expert users (16, 24). For detection of GAD67-positive somata, we modified the previously reported automated algorithms to allow for reliable detection independent of the density of GAD67-positive boutons. The modifications are described in detail in *SI Appendix*. Note that in columns A1–3 and B2–3 of one animal, uneven staining prevented automatic separation between GAD67-positive and -negative somata in some portions of supragranular and granular layers. Excitatory/inhibitory neuron numbers of A1–3 and B2–3 in S1 (Tables 1; *SI Appendix*, Tables S3 and S4) are thus based on data from three, instead of four, animals.

Section Alignment. Outlines of anatomical structures (i.e., pia, barrels, and WM in cortex, and VPM, barreloids, and RT in thalamus) were manually drawn on median projections of the GAD67 image stacks for each section. Using the blood vessel patterns as reference landmarks, the contours and soma landmarks of adjacent cortical brain sections were aligned manually by rigid transformations using ZIBAmira software (39), as reported previously (23). Contours and soma landmarks from thalamic sections were aligned analogously using the outlines of RT and individual barreloids as reference structures.

Cortical and Thalamic 3D Reference Volumes. The 3D reconstruction of cortex geometry was performed as described previously (23). Briefly, barrel outlines and orientations were reconstructed in 3D from the 2D contours. Barrel

columns were reconstructed by extrapolating the L4 barrel outlines along the vertical column axes toward the pia and WM surfaces (compensating for overlapping columns in deep layers) (23). The border between vibrissal cortex and DZ was defined based on the convex hull around all reconstructed barrel columns. Reference volumes in the DZ were selected manually and, on average, comprised volumes of ~ 2.5 mm³. Barreloids in VPM thalamus were reconstructed from outlines using 3D Delaunay triangulation. Reference volumes in P0m were selected manually and, on average, comprised volumes of ~ 0.4 mm³.

Neuron Densities and Absolute Neuron Numbers. Soma distributions were scaled to a slice thickness of 50 μ m. Each neuron soma was assigned to the closest barrel column/barreloid or to septum, as well as to supragranular, granular, or infragranular cortical layers (i.e., above, within, or below the L4 barrels). Total neuron counts for individual columns/barreloids were obtained by summing up all neuron somata assigned to the same column/barreloid. Vertical density profiles were computed in 50- μ m steps along the respective vertical column axes. Density profiles along the row or arc were obtained by registration to a standardized model of rat vibrissal cortex (23).

Statistical and Analysis Routines. If not mentioned otherwise, all computations were performed using custom-written software with the Insight Segmentation and Registration Toolkit and Visualization Toolkit libraries in C++ (40).

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