Honeycomb-Like Mosaic at the Border of Layers 1 and 2 in the Cerebral Cortex

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In this report, we present evidence of a small-scale modularity (<100 μm) at the border of layers 1 and 2 in neocortical areas. The modularity is best seen in tangential sections, with double-labeling immunohistochemistry to reveal overlapping or complementary relationships of different markers. The pattern is overall like a reticulum or mosaic but is described as a “honeycomb,” in which the walls and hollows are composed of distinct afferent and dendritic systems. We demonstrate the main components of the honeycomb in rat visual cortex. These are as follows: (1) zinc-enriched, corticocortical terminations in the walls, and in the hollows, thalamocortical terminations (labeled by antibody against vesicular glutamate transporter 2 and by cytochrome oxidase); (2) parvalbumin-dense neuropil in the walls that partly colocalizes with elevated levels of glutamate receptors 2/3, NMDAR receptor 1, and calbindin; and (3) dendritic subpopulations preferentially situated within the walls (dendrites of layer 2 neurons) or hollows (dendrites of deeper neurons in layers 3 and 5). Because the micromodularity is restricted to layers 2 and 1b, without extending into layer 3, this may be another indication of a laminar–specific substructure at different spatial scales within cortical columns. The suggestion is that corticocortical and thalamocortical terminations constitute parallel circuits at the level of layer 2, where they are segregated in association with distinct dendritic systems. Results from parvalbumin staining show that the honeycomb mosaic is not limited to rat visual cortex but can be recognized at the layer 1–2 border in other areas and species.

Key words: columnar organization; zinc-enriched corticocortical terminals; thalamocortical terminals; parvalbumin; dendritic minicolumn; rat visual cortex; cytochrome oxidase

Introduction
Vertical modularity is one of the defining characteristics of cortical structures, but the specific features are not identical across areas. In primary sensory areas, a conspicuous modularity has been associated with thalamocortical (TC) terminations, especially in layer 4 (Lund, 1988; Chmielowska et al., 1989; DeFelipe and Jones, 1991; Casagrande and Kaas, 1994; Rockland, 1998), but in limbic areas, where layer 4 is absent or poorly developed, it is the superficial layers that have a pronounced modularity (Amaral et al., 1987; Kostovic et al., 1993).

In a previous study of rat granular retrosplenial cortex (Ichinohe and Rockland, 2002a), we demonstrated a new system of dendritic bundling in layer 1. This originates from GABAergic inhibitory neurons and comingles with apical dendrites of layer 2 pyramidal neurons (Wyss et al., 1990). As a further demonstration of the complex organization of the superficial layers in this area, we noted that the dendritic bundles merge, at the border of layers 1 and 2, into a honeycomb-like structure, with walls consisting of parvalbumin (PV)-dense neuropil.

In this report, we present evidence for a similar small-scale modularity at the border of layers 1 and 2 in neocortical areas. The pattern is overall like a reticulum or mosaic but is described as a honeycomb in which the walls and hollows are revealed by markers for distinct neurochemical substances, afferents, or dendritic subpopulations. Three aspects of the honeycomb provide a new perspective on cortical organization. First, its localization to layers 2 and 1b suggests the particular importance of this stratum, even outside limbic areas. Second, because the micromodularity is restricted to layers 2 and 1b, this is additional evidence for a laminar–specific substructure, at different spatial scales, within the cortical column as traditionally understood in relation to thalamic terminations in layer 4 (for review, see Mountcastle, 1977). Third, the particular composition, as we will show in Results, involves high levels of zinc and NMDA receptor 1 (NMDAR1), substances that have been specifically involved in plasticity effects.

As a first step, we have performed a detailed characterization of the honeycomb in rat visual cortex, using cell type-specific and functionally related markers. PV immunohistochemistry was also used to investigate the occurrence of the honeycomb in other areas and species.

Some of these results have been published previously in abstract form (Ichinohe and Rockland, 2002b).

Materials and Methods
Experimental subjects. Forty-five adult male Wistar rats (>9 weeks old) were used in this study. Blocks from cat (n = 3) and monkey tissues (n = 3) were excised from brains used in other studies. All experimental protocols were approved by the Experimental Animal Committee of the RIKEN Institute and were performed in accordance with the Guidelines.
for the Use of Animals in Neuroscience Research (The Society for Neuroscience).

**Fixation and tissue preparation.** Adult rats were anesthetized with Nembutal (100 mg/kg). They were perfused transcardially, in sequence, with 0.9% saline and 0.5% sodium nitrite, and 4% paraformaldehyde with or without saturated 15% picric acid, in 0.1 M phosphate buffer (PB), pH 7.3. Four rats designated for zinc histochemistry were perfused with 0.1 M PB containing 0.1% sodium nitrite and then 0.1% sodium sulfide and 4% paraformaldehyde. Postfixation in the same fixative was for 2 hr. Then the brains were placed in 30% sucrose and after sinking were cut into 40-μm-thick tangential or coronal sections on a freezing microtome.

**Immunoperoxidase staining for PV.** Sections were incubated for 1 hr with 0.1 M PBS, pH 7.3, containing 0.5% Triton X-100 and 5% normal goat serum (PBS-TG) at room temperature and then for 40–48 hr at 4°C with 0.1 M PB containing 0.1% sodium sulfide and then 0.1% sodium sulfide and 4% paraformaldehyde. Postfixation in the same fixative was for 2 hr. Then the brains were placed in 30% sucrose and after sinking were cut into 40-μm-thick tangential or coronal sections on a freezing microtome.

Figure 1. PV immunoreactivity shows a honeycomb pattern in superficial layers in the rat V1. **A–C,** Serial tangential sections stained by immunoperoxidase method for PV. PV-ir walls surround open hollows (arrowheads). **D,** Higher magnification. **Arrowheads** point to PV-ir terminal-like puncta. **E, F,** Tangential section. Double staining for PV (immunofluorescence, **E**) and Nissl substance (**F**) shows that the hollows tend to contain fewer cells than do the walls. **Arrowheads** point to corresponding spaces. **G, H,** Coronal section stained by immunoperoxidase method for PV. The hollows are less conspicuous but can be detected as a series of notches (arrowheads). **I, J,** Coronal section. Double staining for PV (H) and Nissl substance (**I**) confirms that the honeycomb is located at the level of layer 2. **Arrowheads** point to corresponding hollows. **J–L,** Coronal section. Double-immunofluorescent staining for PV and GAD65 demonstrates colocalization of these two markers, as expected if the small PV-ir particles were GABAergic terminals. Double-labeled structures frequently made basket-like terminations on immunonegative somata (arrows). Scale bar (shown in **A**): **A–C,** 200 μm; **D,** 80 μm; **E–I,** 100 μm; **J–L,** 25 μm.
Measurements and analysis. Quantitative analysis was achieved with the aid of a Neurolucida System (MicroBrightField, Colchester, VT). Tangential sections were selected that had been reacted for PV or CO. The centers of the PV-immunoreactive (PV-ir) honeycomb hollows and CO-positive patches were plotted in an area of at least 120,000 μm² from two tissue sections for each structure, using a 20× objective lens. The nearest center-to-center distance was obtained by using NeuroExplorer analysis software (MicroBrightField). Although many of our figures are shown at lower magnification, we examined all materials using higher magnification (200 or 400×) in an attempt to evaluate nonspecific staining. We note that our observations relate to strong PV-ir labeling. There is some possibility that SOM and PV may colocalize, but this pertains to PV-ir profiles that are weak and has been shown to be very slight in neocortex (Kosaka et al., 1987; Kubota et al., 1994; Gonchar and Burkhalter, 1997).

Identification of areas. Visual cortex in the rat has been divided into subfields according to several criteria and nomenclatures (Montero, 1981; Malach, 1989; Zilles, 1990; Paxinos and Watson, 1998; Rumberger et al., 2001). In this report, we concentrate on area V1, both monocular and binocular subdivisions, and use the terminology of Paxinos and Watson (1998). No obvious difference between subdivisions of V1 was noticed as regards the honeycomb, and we describe results from both regions. In coronal sections, posterior to the dentate gyrus, we identified area V1 at lateral anterior 3.0 and lateral 2.0–5.0. Other areas are also identified according to Paxinos and Watson (1998).

In addition to guidance from the published literature, we verified histologically that we were within area V1. Both AchE and MBP showed clear changes in layers 4 and 5 at the lateral and medial borders of area V1 (Zilles et al., 1984).

Identification of layers 1 and 2 (rat visual cortex). As frequently described, layer 1 is a conspicuously cell-sparse zone (~150 μm wide) in Nissl stains. In myelin preparations, however, a thin, myelin-dense outer zone, layer 1a (~70 μm wide), can be distinguished from a myelin-sparse inner zone, layer 1b (~80 μm wide). A thin, outermost part of layer 1a again appears myelin sparse, but this is not considered separately in this report. In Nissl stains, layers 2 and 3 are frequently not distinguished. However, on the basis of our results, we have delineated layer 2 as a distinct zone, ~100–120 μm in thickness, subjacent to layer 1.

Results

The honeycomb structure can be demonstrated by several markers with overlapping or complementary relationships. Because PV [which stains a subpopulation of GABAergic cortical interneurons (Kosaka et al., 1987; Kubota et al., 1994; Gonchar and Burkhalter, 1997)] reliably and strongly shows the honeycomb pattern, and because the reaction protocol is easily compatible with other markers, PV is used as the standard reference for double labeling throughout this study. In the first part of Results, therefore, we report the basic structure of the honeycomb as demonstrated by PV. In the subsequent sections, we consider several afferent and other neuronal components, which may be part of a specialized circuitry. Some of these, especially as shown by zinc and VGluT2,
extend into layer 1b. At the end of Results, we discuss other markers, some of which seem not to conform to either the walls or hollows, and present preliminary evidence that the honeycomb occurs in other regions and other species.

**Honeycomb shown by PV**

Tangential sections stained for PV clearly reveal a honeycomb-like organization, with distinct walls and hollows in the superficial layers of rat V1 (Fig. 1A–E). The walls of the honeycomb consist of PV-ir small, fine, terminal-like particles and PV-ir cell bodies, fibers, and dendritic elements (Fig. 1D, F). In serial tangential sections, each honeycomb compartment is recognizable in one to three sections; that is, through ~120 μm (section thickness = 40 μm). Below this, the honeycomb merges into a homogeneously dense PV-ir neuropil (Fig. 1A–C). We define layer 2 as the layer with PV-ir honeycomb pattern, and comparison with Nissl stains (for cell bodies) confirms a slightly increased cell density at this level, relative to layer 3 (Fig. 1H, I). The center-to-center spacing of the hollows is ~80 μm (mean value) and ranges from 50 to 120 μm. Although the honeycomb pattern is discerned most easily in tangential sections, it can also be recognized in radial sections, where the hollows appear as a series of notches (Fig. 1G). Double staining for PV and Nissl substance shows that the hollows tend to contain fewer cells than the walls (Fig. 1E, F, H, I).

The identity of the terminal-like particles was investigated by staining for GAD65, which is localized in GABAergic terminals (Erlander et al., 1991). Double-immunofluorescent staining for PV and GAD65 demonstrates colocalization of these two markers (Fig. 1J–L), as would be expected if the small PV-ir particles were GABAergic terminals. More particularly, double-labeled structures frequently made basket-like terminations on immunonegative somata (Fig. 1L), consistent with classical depictions of PV basket endings.

**Excitatory terminal markers**

Next we investigated how other terminations, especially excitatory terminations, are related to the PV honeycomb. For this purpose, we used zinc and VGluT2 as general markers for two distinct connectional systems. Zinc is known to distinguish a subpopulation of corticocortical (CC) excitatory terminals (Slomianka et al., 1990; Garret et al., 1992; Casanovas-Aguilar et al., 1998, 2002). VGluT2, in contrast, is a marker for TC terminals (Fujiyama et al., 2001; Kaneko and Fujiyama, 2002; Kaneko et al., 2002). Histochemistry for zinc in tangential sections shows fine zinc-positive small particles in a distinct honeycomb pattern (Fig. 2A). As established by double labeling, the zinc pattern closely colocalizes with the PV-ir walls in layer 2 (Fig. 2B, C). The zinc walls, however, are taller and extend into layer 1b (Fig. 2B–F). In coronal section, zinc-negative honeycomb hollows appear as gaps, or notches, of reduced labeling, and the greater height of the walls, extending into layer 1b, can be discerned easily (Fig. 2D, E). In layer 1a, zinc staining is very weak, and below layer 2, it also becomes gradually weaker.

Immunohistochemistry for VGluT2 shows uniformly dense staining in layer 1a but a discontinuous, periodic pattern in layers 1b and 2. With double labeling for VGluT2 and PV, the VGluT2-ir dense regions in layer 2 can be seen to lie within the PV hollows (Fig. 3). One can infer, therefore, that VGluT2-ir TC terminals are complementary to zinc-enriched CC terminals in layers 1 and 2. Both periodic systems extend into layer 1b, higher than the PV honeycomb walls (Fig. 3D–F). Below layer 2, immunoreactivity for VGluT2 becomes stronger through the deeper part of layer 3, where narrow vertical bundles, possibly corresponding to ascending axons, are frequently in evidence.

**Cell body and neuropil markers**

The complementary relationship between zinc and VGluT2 strongly supports the possibility that the honeycomb is a distinct specialization in the upper layers. To further characterize this structure, we screened additional markers suitable for visualizing putative neuronal subpopulations or postsynaptic structures. These are GluR2/3, a marker of excitatory pyramidal neuronal
cell bodies and dendritic shafts and spines (Petraila and Wenthold, 1992; Gutierrez-Ibarluzea, 1997); NMDAR1, expressed by 80% of cortical neurons (Conti et al., 1994), including many with the morphological features of pyramidal neurons (Aoki et al., 1994); CB, a marker, in rat cortex, for GABAergic and some pyramidal neurons (Celio, 1990; Gonchar and Burkhalter, 1997); MAP2, a marker for thick apical dendrites (Escobar et al., 1986); GABAa receptors (Benke et al., 1991); and GABAa subunit, contained in 80% of cortical neurons (Conti et al., 1994), including many with the morpho-

dritic markers
Other prominent components of the superficial layers are pyramidal cell apical dendrites. In fact, a distinct organization of apical dendrites has been reported previously by several laboratories using MAP2 antibody (Escobar et al., 1986; Peters and Sethares, 1991a; Peters and Yilmaz, 1993). These studies report that MAP2-ir apical dendritic shafts of pyramidal neurons in layers 3 and 5 form distinct bundles, which are about the same dimension (<100 μm) as the layer 2 honeycomb. By analysis of coronal and tangential sections, we confirmed that MAP2-ir dendrites form distinct bundles, but we would further suggest that there are several subpopulations.

First, double labeling for MAP2 and PV indicates bundles of strongly MAP2-ir apical dendrites that are contained primarily in honeycomb hollows (Fig. 7A–I). In serial sections, the thick dendritic bundles in the hollows can be followed down to layer 5.

GluR2/3 immunoreactivity reveals a distinct honeycomb pattern in layers 1b and 2 (Fig. 4A). Double labeling for PV shows that the regions dense for GluR2/3 colocalize with the PV dense walls, although as with zinc, these extend higher into layer 1b (Fig. 4A–C). As expected from Nissl staining, GluR2/3-immunopositive cell bodies in layer 2 (presumably excitatory pyramidal neurons) are concentrated in the walls (Fig. 4D–F). The GluR2/3 label extends into layer 1b, where it is probably composed of apical dendritic components of layer 2 pyramidal neurons. Most GluR2/3 cell bodies are surrounded by PV-ir basket-like puncta (Fig. 4D–I), consistent with other reports of PV-ir terminals surrounding pyramidal cell bodies. Below layer 2, GluR2/3 immunoreactivity is about as dense as in the honeycomb wall but is overall diffuse, with no indication of periodicity.

Immunoreactivity for NMDAR1 shows a periodic pattern that, after double labeling, is seen to correspond well with the PV honeycomb (Fig. 5A–C). The walls are composed of NMDAR1-ir cell bodies, along with proximal dendrites and fine particles (Fig. 5D–I). In coronal sections, many of the NMDAR1-ir cells have the appearance of pyramidal neurons (Fig. 5G), and none are double labeled for PV. NMDAR1-ir cell bodies, like GluR2/3-ir cells, are frequently surrounded by PV-ir basket-like terminations (Fig. 5D–I), and their proximal dendrites also seem to be targeted by PV-ir puncta (Fig. 5F, I). In our material, no particular NMDAR1-ir pattern is discernable in layer 1; however, Johnson et al. (1996, their Fig. 1b), using an antibody against NMDAR1-C1-splice variant, illustrate distinct protrusions into layer 1b, similar to our results with zinc. Below layer 2, the number of NMDAR1-ir cell bodies is sparser than in layer 2.

CB immunoreactivity demonstrates a honeycomb pattern closely similar to that of PV (Fig. 6A–F) but extending into layer 1b. The CB walls in layer 2 consist of many weakly positive neurons (probably pyramidal neurons) (Kubota et al., 1994), with a few strongly positive neurons (presumably GABAergic) (Fig. 6D–F). Some CB-ir neurons are double labeled for PV. Both the weakly and strongly CB-ir neurons are associated with PV-ir puncta (Fig. 6G–I). In layer 1a, CB staining is very weak, and below layer 2, CB staining becomes gradually weaker (Fig. 6D), similar to zinc staining.

In summary, GluR2/3, NMDAR1, and CB, all of which stain cell bodies and proximal dendrites, have a periodic pattern that is systematically related to the PV-ir honeycomb (but extends higher into layer 1b than does the PV-ir staining).

Figure 5. Immunoreactivity for NMDAR1 shows a periodic pattern that, after double labeling, is seen to correspond well with the PV honeycomb (Fig. 5A–C). The walls are composed of NMDAR1-ir cell bodies, along with proximal dendrites and fine particles (Fig. 5D–I). In coronal sections, many of the NMDAR1-ir cells have the appearance of pyramidal neurons (Fig. 5G), and none are double labeled for PV. NMDAR1-ir cell bodies, like GluR2/3-ir cells, are frequently surrounded by PV-ir basket-like terminations (Fig. 5D–I), and their proximal dendrites also seem to be targeted by PV-ir puncta (Fig. 5F, I). In our material, no particular NMDAR1-ir pattern is discernable in layer 1; however, Johnson et al. (1996, their Fig. 1b), using an antibody against NMDAR1-C1-splice variant, illustrate distinct protrusions into layer 1b, similar to our results with zinc. Below layer 2, the number of NMDAR1-ir cell bodies is sparser than in layer 2.

CB immunoreactivity demonstrates a honeycomb pattern closely similar to that of PV (Fig. 6A–F) but extending into layer 1b. The CB walls in layer 2 consist of many weakly positive neurons (probably pyramidal neurons) (Kubota et al., 1994), with a few strongly positive neurons (presumably GABAergic) (Fig. 6D–F). Some CB-ir neurons are double labeled for PV. Both the weakly and strongly CB-ir neurons are associated with PV-ir puncta (Fig. 6G–I). In layer 1a, CB staining is very weak, and below layer 2, CB staining becomes gradually weaker (Fig. 6D), similar to zinc staining.

In summary, GluR2/3, NMDAR1, and CB, all of which stain cell bodies and proximal dendrites, have a periodic pattern that is systematically related to the PV-ir honeycomb (but extends higher into layer 1b than does the PV-ir staining).

Dendritic markers
Other prominent components of the superficial layers are pyramidal cell apical dendrites. In fact, a distinct organization of apical dendrites has been reported previously by several laboratories using MAP2 antibody (Escobar et al., 1986; Peters and Sethares, 1991a; Peters and Yilmaz, 1993). These studies report that MAP2-ir apical dendritic shafts of pyramidal neurons in layers 3 and 5 form distinct bundles, which are about the same dimension (<100 μm) as the layer 2 honeycomb. By analysis of coronal and tangential sections, we confirmed that MAP2-ir dendrites form distinct bundles, but we would further suggest that there are several subpopulations.

First, double labeling for MAP2 and PV indicates bundles of strongly MAP2-ir apical dendrites that are contained primarily in honeycomb hollows (Fig. 7A–I). In serial sections, the thick dendritic bundles in the hollows can be followed down to layer 5.
From layer 5, two dendritic bundles frequently merged together to form a single bundle within one honeycomb hollow. Apical dendrites from layer 3 could be seen joining with these deeper bundles, as reported previously (Escobar et al., 1986; Peters and Sethares, 1991a; Peters and Yilmaz, 1993). Smaller bundles of thick apical dendrites are less strictly confined within the hollows. A second subpopulation consists of apical dendrites of neurons in the honeycomb walls (in layer 2). These are thinner dendrites, closer to the pia surface, and less frequently labeled by MAP2. Their origin from superficial neurons, which are not stained by MAP2, can be inferred by double labeling for MAP2 and PV, because the position of cell bodies is rendered visible by PV-ir basket-like terminations (Fig. 7D–F). With MAP2 staining for dendrites, therefore, the separation between walls and hollows is not as clear as with PV and other markers.

In layer 1b, MAP2-ir dendrites in the hollows bifurcate, becoming thinner, and the distal apical tufts frequently arch over the honeycomb walls (Fig. 7D–F). In contrast with VGluT2, MAP2 immunohistochemistry does not show any distinct pattern in layer 1b.

GABAα1 is a general neuropil marker but often yields an image of vertically oriented, presumably dendritic processes. In our material, immunoreactivity for GABAα1 shows a distinct periodicity that, especially in tangential sections, has a honeycomb configuration. After double labeling with PV, regions high in GABAα1, similar to VGluT2, can be seen to coincide with PV hollows (Fig. 8A–F). Bundles of GABAα1-ir dendrite-like structures in layer 2 extend into layer 1b before merging into a uniform neuropil (Fig. 8D). Interestingly, in development, the strong expression of GABAα1 is well matched with thalamically innervated layers (layers 1, 3, and 4) in V1 and S1 (Paysan et al., 1994) and is dependent on thalamic innervation (Paysan et al., 1997).

GluR5/6/7 immunohistochemistry stains thick apical dendrites strongly and cell somata weakly (data not shown). The overall staining pattern in layer 2 is similar to MAP2 immunoreactivity. That is, immunoreactivity is higher in the PV hollows, without any distinct pattern in layer 1. Faintly immunopositive neurons are found in honeycomb walls, and some neurons are also double labeled for PV and GluR5/6/7.

**Other neuronal markers**

Because PV demarcates subpopulations of GABAergic neurons, it seemed important to screen for markers of other GABAergic subpopulations (i.e., SOM and CR) (Kosaka et al., 1987; Kubota et al., 1994; Gonchar and Burkhalter, 1997). Reacting for SOM does not result in any obvious pattern in layers 1 or 2 (data not shown). This is not surprising because SOM-ir neurons are not common at this level, but neither did CR exhibit any particular pattern in relationship to PV walls or hollows (Fig. 9A–E). CR-ir cell bodies are only weakly targeted by PV-ir terminations, judging from the apparent absence of basket-like specializations (Fig. 9F).

We also examined classical histochemical markers, such as CO, AChE, NADPH-d, and myelin staining (by immunohistochemistry for MBP). Staining for AChE, NADPH-d, and MBP does not show any honeycomb pattern in layers 1 and 2. CO staining, however, shows a faintly patchy pattern in layers 1b and 2, in tangential sections (Fig. 10A, B). Double histochemistry for CO and PV shows that CO activity is preferentially elevated in the PV honeycomb hollows, although continuing into layer 1b (Fig. 10B, C). The CO patches are about the same size as PV-ir honeycomb hollows (center-to-center distance, mean 80 μm; range, 50–124 μm; measurements are from layer 2 where the patches are most conspicuous.). In layer 1a, the CO patches merge into denser diffuse staining, as in VGluT2-ir (Fig. 10A, B). In other thalamic recipient layers in V1 (i.e., layers 4 and 6a and the lower part of layer 3), CO activity is differentially higher but does not exhibit any periodicity.

**Other area and other species**

To test whether the honeycomb specialization is peculiar to rat visual cortex, we screened several additional areas for PV. Sections reacted for PV show a honeycomb mosaic in area V2, adjacent to area V1. Tangential sections through barrel cortex and medial prefrontal area show a distinct honeycomb configuration in layer 2 (Fig. 11A, B). In
barrel cortex, the honeycomb is confined to layer 2, without continuing into layer 3, and is of a smaller scale than the underlying barrels in layer 4 (≈80 vs. ≈300 μm center-to-center distance). The honeycomb structure can be recognized in other species, as seen by PV staining: cat area 17 and several areas in monkey (Fig. 11C-D). There are slight differences in size and distinctness, and additional work is necessary to clarify areaspecific patterns of overlapping and complementary relationships of different substances, as shown for rat visual cortex.

Discussion

Our results demonstrate a reticulated or honeycomb-like organization at the border of layers 1 and 2. The term “honeycomb” may be deceptive, because the organization is not strictly regular; however, it conveys the small-scale dimension and the complementary, wall-and-hollow pattern as seen with single labels. The main components of the honeycomb in rat visual cortex are as follows: (1) segregated afferent systems that can be attributed to CC (zinc) and TC (VGluT2) terminations; (2) PV-dense walls that colocalize with elevated levels of GluR2/3, NMDAR1, and CB; and (3) dendritic systems at least partially segregated to the walls (dendrites of layer 2 neurons) and hollows (dendrites of deeper neurons in layers 3 and 5) (Fig. 12). The implication is that CC and TC circuitries are to some extent two parallel systems at the level of layer 2. The CC terminations may differentially target the dendrites of layer 2 pyramids, and TC terminations may target the distal apical dendrites of deeper neurons in layers 3 and 5. Some elements, however, such as CR-ir neurons, do not strictly fit the honeycomb pattern but perhaps have a separate organization in layer 2. Because the honeycomb-like pattern is also observed, in a different degree of distinctness, in other areas and other species, this small-scale structure in layer 2 may be a basic feature of neocortical organization.

Comparison with other work

Although it is surprising that such a conspicuous structure has not been reported...
previously, the honeycomb pattern can easily be missed. This is because of its thinness and the fact that it is best seen in serial tangential sections, with double-labeling to reveal overlapping or complementary patterns. In retrospect, however, a periodic tangential structure can be discerned in many published images [e.g., Nissl staining (Peters and Kara, 1985, their Fig. 18); PV and CB (Célio, 1990, their Fig. 9G,H); zinc (Perez-Clausell, 1996, his Fig. 15); MAP2 (Schmolke and Kunzle, 1997, their Fig. 2b); NMDAR1 C1 splice variants (Johnson et al., 1996, their Fig. 9B)].

Among the honeycomb components, one of them, the apical dendritic bundles, has in fact been described by other investigators. These studies reported that pyramidal neurons in layers 3 and 5 form compact bundles of apical dendrites [mouse (Escobar et al., 1986); rat (Peters and Kara, 1987); rabbit (Schmolke and Viebahn, 1986); cat (Peters and Yilmaz, 1993); monkey (Peters and Sethares, 1991a)] and further discussed whether these might be substrates of cortical minicolumns (Peters, 1994; Mountcastle, 1997). Our results demonstrate similar apical dendritic bundles (within hollows, where several bundles may merge together), but we emphasize that these are only one of several dendritic systems. Notably, another population is made up of smaller caliber dendrites of layer 2 pyramidal neurons. These seem to remain spatially segregated within honeycomb walls, where they overlap with zinc-enriched, putative CC inputs and with concentrations of PV-positive neuropil (Fig. 12).

The segregation of different dendritic systems is accentuated specifically at the level of the honeycomb in layers 1b and 2. In upper layer 1, there is considerable intermingling of the dendritic neuropil, and in layer 3, thick dendritic bundles continue, but not within the honeycomb organization. Yet other distinct subpopulations may be distinguishable; for example, dendrites of layer 6 neurons were identified as being separate by earlier investigations (Escobar et al., 1986; Peters and Kara, 1987).

One implication of these results is that in neocortex, as in limbic cortices, layer 2 has a distinct and prominent role. Our recent study of the periallocortical granular retrosplenial region in rats identified a honeycomb organization in layer 2, which is continuous with laterally adjacent areas (Ichinohe and Rockland, 2002a). More work is necessary, however, to determine regional and species differentiation.

A second implication is that there is a significant laminar-specific substructure across the cortical thickness. Anatomically, it is well established that cortical columns, when considered as 300- to 500-μm-wide structures (equivalent to TC domains in layer 4), are not homogeneous. For example, in primate visual cortex, TC terminations in layers 3 (the CO patches), 4A, 4Ca, 4CB, and 6 are all at different spatial scales (Lund, 1988; Casagrande and Kaas, 1994). Physiological studies also report laminar-specific differences in magnification factors (Blasdel and Fitzpatrck, 1984; Tootell et al., 1988a). In the case of the layer 2 honeycomb, it is not clear whether this is best regarded as a closely integrated subdivision of a pia-to-white matter "column" or, perhaps more likely, whether it might be operating in parallel with a TC unit, based in layer 4. One might even suppose that layer 4 is primarily concerned with topographic, local operations, whereas layer 2 might operate as a widespread network [see also Jones (1998) on topographically specific and diffuse TC inputs to layers 4 and 1, respectively].

In scale, the layer 2 honeycomb is very similar to the layer 4A honeycomb in primate area V1, but in the latter, thalamic afferents coincide with PV-ir and CB-ir neuropil in the walls (Peters and Sethares, 1991b). The hollows are reported to contain pyramidal cell clusters displaced from layer 4B (Peters and Sethares, 1991b; Hendry and Bhandari, 1992; Preuss and Coleman, 2002).

The alternation of TC and CC afferents in the layer 1–2 honeycomb conforms to a common feature of cortical modularity that is believed, in general, to subserve parallel processing. A close comparison is with the rat barrel field, where the cell-dense barrels receive thalamic afferents from the ventral posterior medial nucleus and have short-range intrinsic connections, whereas the
inter-barrel septa receive afferents from the posterior medial thalamus, have long-range intrinsic connections, and have more extensive extrinsic connections (Kim and Ebner, 1999). The CO patches in cat and primate are another natural comparison. These are known to match with direct thalamic inputs, and with high GABAa/ir immunoreactivity, but to be complementary with zinc, like the layer 2 honeycomb (Hendry et al., 1990; Dyck and Cynader, 1993; Murphy et al., 1995). Unlike the honeycomb, however, no dendritic bundling or stratification has been associated with these deeper CO blobs.

How does the organized arrangement of TC and CC inputs in layer 2 relate to dendritic targets? Although more work is necessary here also, the implication is of a high degree of connectional selectivity. In layer 2, TC terminations target the distal apical tufts of deeper pyramidal neurons, whereas CC inputs preferentially colocalize with apical dendrites of superficial pyramidal neurons and with PV-ir neuropil. In layer 1a, however, both dendritic populations in their distalmost portions are accessible to TC but not to zinc-enriched CC terminations. Stratification along the distal dendrites recalls the piriform cortex, where distal apical tufts of pyramidal neurons in layers 2 and 3 receive stratified inputs. These are from extrinsic olfactory afferents, in layer 1a, and from zinc-enriched cortical association inputs, in layer 1b (Haberly, 1998). Similarly, in vitro results have indicated that local connections of interneurons and pyramidal neurons are not probabilistic or random but rather highly selective (for review, see Silberberg et al., 2002).

Functional significance
Is the honeycomb in rat visual cortex expressly related to visual processing? It may be relevant in this regard that laminar differences have been reported in response to spatial frequency. In particular, layer 2 neurons in the rat have a higher spatial frequency preference than do neurons in the deeper layers (Girman et al., 1999). According to our results, layer 2 neurons are likely to be situated in the CO-weak honeycomb walls. This would agree with results, in both monkeys and cats, in which neurons in the CO blobs (our “hollows”) preferentially respond to low spatial frequencies, and those located in interblobs (our “walls”) respond to high spatial frequencies (Tootell et al., 1988b; Born and Tootell, 1991; Shoham et al., 1997).

One might speculate that the honeycomb may be related to developmental phenomena. That is, in early neonate, optical recordings of brain slices labeled with fluorescent calcium indicator reveal distinct domains of spontaneously coactive neurons, coupled by gap junctions (Yuste et al., 1992; 1995; Peinado et al., 1993). The domain size (50–120 μm) is very similar to the honeycomb spacing. Paysan et al. (1994) demonstrated aggregates (80–120 μm) in layer 3 of GABAa/ir pyramidal neurons and dendrites, again of dimensions similar to the GABAa/ir dendritic bundles in the adult honeycomb.

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Figure 11. A honeycomb-like mosaic can be seen in layer 2 in other areas and other species. Tangential sections reacted for PV by immunoperoxidase method. A, Rat barrel cortex. B, Rat medial prefrontal cortex. C, Cat visual cortex, D, Monkey primary auditory cortex. Scale bar, 200 μm.

Figure 12. Highly schematic summary of the micromodularity of layers 1 and 2 of the rat visual cortex. Honeycomb walls in layer 2 (darker shading) are preferentially occupied by somata and proximal dendrites of layer 2 pyramids. These comingle with PV-ir GABAergic terminals and zinc-enriched corticocortical terminals. The zinc-enriched terminals extend into layer 1b, where they are likely to target the dendrites from layer 2 pyramidal neurons. Apical dendrites of deeper pyramidal neurons preferentially occupy honeycomb hollows (lighter shading) and colocalize with VGLUT2-ir thalamocortical terminals. Other markers related to the honeycomb mosaic are listed. The degree of segregation and precise microcircuitry organization will need to be investigated further.
hollows observed in the present study. Finally, it is interesting to note the higher levels of zinc and NMDAR1 within the honeycomb, substances that have been consistently associated with plasticity (Bear, 1996; Li et al., 2001; Brown and Dyck, 2002).

References
