Neurons Immunoreactive for Vasoactive Intestinal Polypeptide in the Rat Primary Somatosensory Cortex: Morphology and Spatial Relationship to Barrel-Related Columns

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ABSTRACT

Vasoactive intestinal polypeptide (VIP) in neocortex affects neuronal excitability as well as cortical blood flow and metabolism. Interneurons immunoreactive for VIP (VIP-IR neurons) are characterized by their predominantly bipolar appearance and the radial orientation of their main dendrites. In order to determine whether the morphology of VIP-IR neurons is related to the functional organization of the cortex into vertical columns, we combined both immunostaining of neurons containing VIP and cytochrome oxidase histochemistry for visualizing barrels, morphological layer IV correlates of functional columns, in the primary somatosensory (barrel) cortex of rats. VIP-IR neurons were localized in supragranular (48%), granular (16%), and infragranular layers (36%) as well as in the white matter. In the granular layer, a clear trend that more neurons were located in interbarrel septa rather than in barrels could be observed, resulting in a neuronal density which was about one-third higher in the septal area. VIP-IR neurons from the different cortical layers were three-dimensionally reconstructed from serial sections by using a computer microscope system. The neurons were mostly bipolar. Striking morphological differences in both axonal and dendritic trees were found between neurons whose cell bodies were located in supragranular, granular, and the upper part of infragranular layers, and those whose cell bodies were located in the area below. The former had dendrites which often reached layer I, where they bifurcated several times, and axonal trees which were particularly oriented vertically, with a tangential extent smaller than the width of barrels. Therefore, these neurons were mostly confined to either a barrel- or septum-related column. By contrast, the dendrites of neurons of the latter group did not reach the granular layer. Furthermore, these neurons had axons with sometimes very long horizontal collaterals, which often spanned two, in one case three, barrel columns. It is proposed that the differential morphology of neurons with different locations as stated above parallels to some extent the divergence of input streaming into the corresponding layer-defined areas. As a possible consequence of this, VIP-IR neurons may be capable of adapting the excitability and metabolism of cortical compartments either in a spatially limited or more extensive way. J. Comp. Neurol. 420:291–304, 2000. © 2000 Wiley-Liss, Inc.

Indexing terms: VIP; GABA; barrel cortex; modular organization; cortical metabolism
Vasoactive intestinal polypeptide (VIP) in mammalian neocortex is mostly expressed by neurons of a striking bipolar morphology, having a fusiform soma and a very small number of dendrites, very often only one, emanating from each pole. Due to the prominent radial orientation of the dendrites and their vertical extent through several cortical layers (Connor and Peters, 1984; Morrison et al., 1984; Hajós et al., 1988), these neurons have the potential for receiving and integrating input from various afferent systems targeting these layers. For example, direct thalamocortical afferents to VIP-immunoreactive (VIP-IR) neurons in the rat primary somatosensory cortex have been shown to exist, indicating a possible role of these neurons in the early modulation of the cortical processing of afferent sensory input (Staiger et al., 1996a). It is conceivable that the somatodendritic morphology of VIP-IR neurons is related to the functional organization of the neocortex in vertical columns. Each column contains neurons which show similar stimulus-specific response patterns, suggesting a close functional relationship to each other (Mountcastle, 1984). The rat primary somatosensory cortex contains morphological structures, the so-called barrels, which represent layer IV correlates of the functional phenomenon of columnarity (Woolsey and van der Loos, 1970; Welker and Woolsey, 1974). Many experiments have provided evidence that each barrel-related column receives the strongest input from the corresponding mystacial vibrissa of the contralateral whisker pad on the snout of the animal, largely maintaining somatotopy (Welker, 1971; Simons, 1978; Chapin, 1986; Armstrong-James and Fox, 1987; Welker et al., 1993). Therefore, the primary somatosensory cortex of rats is highly suitable for investigating the relationship between the morphology of VIP-IR neurons and their location with respect to functional columns, as already done for spiny and smooth stellate cells in layer IV (Woolsey et al., 1975; Steffen and van der Loos, 1980). Zilles et al. (1993) provided an initial description of the distribution of VIP-IR structures with regard to the barrel architecture in mice. They showed this distribution to be nonrandom. However, the specific contribution of individual neurons to this remained unclear.

VIP is released in a calcium-dependent manner (Martin and Magistretti, 1989); its receptors have been found on neurons, astrocytes, and brain vessels (Martin et al., 1992). Various metabolic and neurotrophic effects have been attributed to VIP, such as an increase of cyclic adenosine monophosphate (cAMP) levels and glycogenolysis in cortical cells (Magistretti and Schorderet, 1984), potentiation of glutamate-dependent expression of brain-derived neurotrophic factor (BDNF) (Pellegrini et al., 1998), promotion of embryonic growth and neuron survival (Breneman and Eiden, 1986; Pincus et al., 1990; Gressens et al., 1997), as well as an increase of cerebral blood flow (Heistad et al., 1980; McCulloch and Edvinsson, 1980). Furthermore, VIP modulates the effects of neurotransmitters (Sessler et al., 1991; Haas and Gähwiler, 1992; Murphy et al., 1993). It seems to do this in synergism with other neuromodulatory substances such as noradrenaline and acetylcholine (Sessler et al., 1991; Murphy et al., 1993), the latter of which has been shown to be colocalized in one-third of VIP-IR neurons (Chédotal et al., 1994; Bayraktar et al., 1997). Finally, VIP-IR neurons colocalize the inhibitory neurotransmitter gamma aminobutyric acid (GABA; Kawaguchi and Kubota, 1997; Bayraktar et al., 1997; Porter et al., 1998), indicating that they act as inhibitory interneurons. All this has led to a rising interest in the morphology of the axonal arbors of VIP-IR neurons, as well as in their postsynaptic targets. Previously, camera lucida drawings of VIP-IR neurons from cat primary visual cortex (Wahle and Meyer, 1989), rat primary visual (Connor and Peters, 1984; Morrison et al., 1984, Hajós et al., 1988), and motor cortices (Kawaguchi and Kubota, 1996; Porter et al., 1998), as well as the hippocampus (Acsády et al., 1996), have been published. However, these reconstructions did not permit conclusions about whether the morphology of the neurons was related to their location with respect to a modular organization of the cortex. To our knowledge, no VIP-IR cell reconstructions from the primary somatosensory cortex have been published to date. Therefore, we stained both VIP-IR neurons by using an antiserum against VIP, and barrels, by demonstration of their enhanced cytochrome oxidase activity (Wong-Riley, 1989), in the same serial coronal sections. Applying this technique, we aimed at obtaining a representative sample of VIP-IR neurons as subjects for the computer-aided three-dimensional reconstruction across serial sections, as performed in the present study in adult rat barrel cortex. Preliminary results of the experiments presented here have been published in abstract form (Bayraktar et al., 1998).

MATERIALS AND METHODS

Preparation of the tissue

The experiments presented here have been performed in accordance to the German law for the protection of animal life. Adult male Wistar rats (n = 7; body weight 250–300 g) were anesthetized with an overdose of intraperitoneally injected pentobarbital (150 mg/kg body weight) and perfused through the ascending aorta with 4% paraformaldehyde and 0.2% picric acid in 0.1 M phosphate buffer (PB; pH 7.4). Brains were removed from the skull and cut on a vibratome into serial sections of 50-μm thickness and standard coronal orientation (n = 4). Alternatively, in three brains, a section plane orthogonal to barrel rows was used, which enables a clear distinction between barrels and septa (Welker and Woolsey, 1974). The sections of these brains were later subjected to cytochrome oxidase histochemistry. Following extensive rinsing in PB, the tissue was immersed in a cryoprotective solution (25% saccharose, 10% glycerol in 0.01 M PB) for 1 hour. After freeze-thawing over liquid nitrogen (3 times), the sections were rinsed in PB several times and then transferred to Tris-buffered saline (TBS, 0.05 M, pH 7.4).

Immunohistochemistry

After 45 minutes of preincubation in 10% normal goat serum (Vector, Burlingame, CA; in TBS, pH 7.4), the tissue was exposed to the primary antibody against VIP (raised in rabbit), which was generously provided by Dr. T. Görs (Gulyás et al., 1990). In the present experiments, replacement of the primary antiserum by normal serum or plain buffer resulted in no detectable staining. After 36–48 hours of incubation at 6°C, the sections were rinsed (4 × 15 minutes) with TBS (pH 7.4) and then exposed to the secondary antisera, i.e., biotinylated goat antirabbit (1:100; Vector) for 2 hours. Again the tissue was rinsed in TBS (pH 7.4; 4 × 15 minutes) and then trans-
the OsO4 was removed, and the slices were rinsed in PB.

minded for supragranular and infragranular layers as well as the sep- 

tal area within layer IV.

Cytoskeletal oxidase histochemistry

After immunohistochemistry was completed, the brain slices cut in a plane orthogonal to barrel rows were subjected to cytochrome oxidase histochemistry in order to visualize barrels. This was performed with minor modifications according to the protocol published by Wong-Riley (1979). Briefly, slices were immersed in an incubation solution containing 6 mg cytochrome C, 5 mg DAB, and 444 mg sucrose in 10 ml PB until brown reaction product became visible in a staining intensity compatible with tracing neurons (Fig. 1A).

The DAB staining of the sections was intensified by treating the tissue with a solution containing 1% OsO4 and 7% beta-D(-)glucose in 0.1 M PB. After 45 minutes, the OsO4 was removed, and the slices were rinsed in PB. Dehydration of the tissue in ethanol followed, with uranyl acetate treatment integrated in the 70% ethanol step. After propylene oxide, the tissue was immersed in Durcupan (ACM, Fluka) overnight and finally mounted onto glass slides, coverslipped and cured at 60°C for 16 hours.

Cell mapping

To determine relative areal differences in the distribution of VIP-IR neurons, we counted these neurons in defined areas and calculated their areal density. Therefore, the outlines of the barrel fields on seven sections counterstained with cytochrome oxidase histochemistry were drawn with the aid of a computer microscope system utilizing Neurolucida software (Microbrightfield, Inc., Colchester, VT; Glaser and Glaser, 1990). By using a lens with 10 times magnification, all VIP-IR neurons localized inside of the outlined areas and visible within one focal plane were marked. Afterwards, the outlines of barrels and the borders between supragranular, cytochrome oxidase-delineated barrels and septa and infragranular layers were drawn. The area sizes of the different compartments within barrel cortex, such as supragranular and infragranular layers, barrels and septa, were measured, and the neurons inside these compartments were counted by using the Morph software (Microbrightfield, Inc.). This procedure was repeated twice on the same section, and the mean from each of the three measured compartments was calculated, in order to account for a possible variability in determining the barrel outlines. In order to directly compare the results of this cell count, the number of neurons per square millimeter of the particular section was determined for supragranular and infragranular layers as well as the barrel and septal area within layer IV.

Reconstruction of the neurons

The cytochrome oxidase histochemistry allowed the precise identification of the primary somatosensory cortex. The staining enabled us to distinguish between supragranular, granular, and infragranular layers. Layer IV was defined by the presence of the cytochrome oxidase-positive patches delineating barrels (see Fig. 1A–C). The requirement of serial sections being immunostained for VIP interfered with an accurate identification of cortical layers; however, the comparison with Nissl-stained sections from different animals enabled a sufficiently precise distinction of layers V and VI. In an early phase of this study, neurons were reconstructed from material not stained for cytochrome oxidase activity, with the aid of a drawing tube (n = 12). However, most neurons (n = 51) were reconstructed three-dimensionally with a computer microscope system and Neurolucida 2.1 software (Micro- brightfield, Inc.). As subjects for reconstruction, we selected neurons inside and in close proximity outside of barrel-related columns, in order to determine possible asymmetries of the neurons if they were located near barrel borders. Whenever the neuronal processes being traced were interrupted at the cut surface of the sections, the corresponding parts in the adjacent section were found by using blood vessels as fiducial marks. The numbers of neurons reconstructed from each layer were initially chosen to proportionally reflect the layer-specific distribution of all neurons in our material, as it resulted from the cell mapping described above. In order to evaluate more closely specific features of the neurons in granular and infragranular layers, we finally reconstructed more of these than this scheme would indicate. Altogether, 63 neurons were reconstructed, 27 of which (43%) were located in supragranular layers, 12 (19%) in layer IV, 12 (19%) in infragranular layers, and 1 in the white matter. For evaluating the neurons, the reconstructions were rotated with computer programs, such as the Maxsim simulation software (Tettoni et al., 1996), equivalent to the orientation of tangential sections, in order to display the three-dimensional appearance of the neurons and their relationship to barrel boundaries. Because the graphics output of Neurolucida 2.1 gives a very coarse appearance of traced structures, the images of neuron reconstructions displayed in this paper have been smoothed by using CorelDraw! 6.0 software (Corel Corporation, Canada) after converting the original data to a metafile format, in order to achieve a genuine representation.

RESULTS

Cell distribution

Our staining protocol resulted in both clearly visible VIP-IR neurons and sufficiently intense cytochrome oxidase (CO) patches and stripes (Fig. 1A–C), indicative of barrels and the layer V VI border region, respectively. This type of staining represented the best achievable balance when combining a VIP immunoreaction with a CO histochemistry in the same section in order to trace axonal arbors in relation to cortical columns. VIP-IR neurons were found in virtually all cortical layers, including the white matter. From double-stained sections, altogether 1,818 VIP-IR neurons from all cortical layers were mapped; 48% of these were located in supragranular layers, 16% in layer IV, and 36% in infragranular layers. Of
the neurons in the granular layer, 7% were located inside the barrels, and 9% in the septa. As to the cellular density in each compartment, the highest density of VIP-IR neurons per square millimeter was found in supragranular layers, referred to as 100%. The neuron density in the granular layer was 62% of that in the supragranular layers, with barrels showing a lower density (51%) than the septa (75%) in all three animals tested. Finally, the density was smallest in infragranular layers (44%). VIP-IR neurons were reconstructed in layers II–VI and the white matter. Figure 1D and E shows the distribution of the cell bodies of 38 reconstructed neurons located within and in close proximity to two particular barrel-related columns.

**Morphology of VIP-IR neurons**

The somatodendritic morphology of VIP-IR neurons in rat primary somatosensory cortex was basically the same as has been reported for other cortical areas (see Introduction). The following cell classes were distinguished according to the characteristics of the cell body and the proximal parts of the dendrites (Fig. 2):

1. Bipolar/single-tufted/bitufted neurons. (a) Bipolar neurons showed one principal dendrite emanating from each pole of the soma. (b) Single-tufted neurons were defined as neurons with either at least two dendrites originating from one pole of the spindle-like soma, or with only one strong dendritic stem rising from it and branching in very close proximity of the cell body. (c) Bitufted neurons had both poles of the soma characterized by the dendritic features defined in b.

2. Modified bipolar and tripolar neurons, a heterogeneous class of neurons with three principal dendrites, often conical cell bodies, but nevertheless typical features of bipolar neurons (for details, see Connor and Peters, 1984).

3. Multipolar neurons, with at least four principal dendrites emanating from different sides of the soma.

4. Other neurons not fitting to the above classes, for example, some neurons from infragranular layers.

In our sample, the population of VIP-IR neurons in rat barrel cortex was mostly composed of bipolar/bitufted neurons constituting 76% of the whole population; 14% of all VIP-IR neurons were tripolar or modified bipolar neurons, 7% were multipolar, and 3% had characteristics not matching this classification. Altogether, although VIP-IR neurons exhibited a large variety of appearances (Fig. 2), the dominant cell type was represented by the classical bipolar/bitufted class.

Due to the vertical orientation of their extended dendritic arbor, many VIP-IR neurons spanned several cortical layers with their dendrites. This was true for almost all of the neurons in supragranular and granular layers. Many of them had one or two dendrites originating from the upper, pially oriented pole of the soma, which passed, without branching, through layers II/III to the molecular layer. There they branched a few times and showed large, regularly shaped varicosities (Fig. 3). In contrast, the dendrites emerging from the lower pole of the soma, extending toward the infragranular layers, branched more frequently close to the soma and generally showed a stronger morphological variability. Although dendritic beads were found in all cortical layers, they were by far most frequent and regularly shaped in layer I. Some VIP-IR neurons had few spines loosely distributed on their dendrites, but a clear correlation of dendritic spines to any of the neuron types mentioned above could not be seen.

The axons of VIP-IR neurons emanated either from one of the primary dendrites close to the cell body, or, less frequently, directly from the soma. The main stem of the axon usually maintained a radial course, either ascending or descending (Fig. 2). Most neurons, predominantly in supragranular and infragranular layers, had mainly descending axon trees. As the axonal arbors of neurons in supragranular layers had a long but horizontally restricted course, they resembled to some extent the axons of a “rodent double-bouquet cell,” as described by Somogyi and Cowey (1984). Descending VIP-IR axonal collaterals were seen to closely approach vertically oriented VIP-IR dendrites, engaging in axodendritic bundles with putatively numerous synaptic contacts. This phenomenon was frequently observed in upper infragranular layers, corresponding to layer V. Nevertheless, about 30% of the reconstructed neurons had primarily ascending axons, especially in lower supragranular layers, in the granular layer, and much less frequently in infragranular layers. Some of these latter types of neurons were characterized by a rather constant set of features, which distinguished them from all morphological VIP-IR subtypes so far described in the literature. Their perikaryon was located in the barrel and sometimes in the lower supragranular layers and had two, occasionally three principal dendrites originating from the soma, giving the neuron a radially extended, bipolar appearance (Fig. 4). The main ascending dendrite constantly bifurcated within a distance of 100 μm from the perikaryon, giving rise to branches extending in an oblique direction. The axon originated either near the bifurcation of the dendrites or a little more proximal, ascended and finally made a hairpin-like turn at the vertex of the tree. Altogether, this neuron presented an aspect which may be described as fountain- or weeping willow-like. Due to their axonal arborization pattern, these cells appeared to focus their output to a horizontally restricted but vertically extended region in barrel and supragranular layers (Fig. 4).

Although we did not identify basket cells among the population of VIP-IR neurons (Somogyi and Cowey, 1984; Wahle and Meyer, 1989; Kawaguchi and Kubota, 1996), perisomatic baskets of axon terminals around unlabeled and VIP-labeled perikarya were frequently observed in our material. Occasionally, some of the neurons reconstructed in this study had axon branches which repeatedly formed pericellular arrays of boutons around immunonegative cell bodies (not shown). However, a systematic investigation of the origin of axons forming pericellular nests, though attempted, was impossible, as we did not succeed in retrogradely tracing these axons back to their initial segments due to the incompleteness of staining, probably caused by the myelination of the axons.

**Layer-specific differences, relationship to barrel related columns**

Several characteristics in both dendritic and axonal morphology, associated with a specific laminar location of the cells, led us to distinguish between two large and heterogeneous groups of VIP-IR neurons. The first group contained neurons whose cell bodies lay within layers II/III through upper layer V, whereas the second group consisted of neurons whose somata were located in lower layer V and layer VI. Concerning dendritic arborization,
Fig. 1. Light micrographs and drawings, indicating the extension of barrels, in and around which several neurons have been selected for reconstruction. A: Low-power photomontage of one section through the parietal cortex (Roman numerals, layers I–VI) double-stained for cytochrome oxidase (barrels, delineated with edge symbols) and vasoactive intestinal polypeptide-immunoreactive (VIP-IR) neurons. Arrows point to neurons that are shown in detail in the following figures with corresponding numbers. B,C: Low-power photomontages from sections adjacent to the one shown in A, demonstrating the continuation of the marked barrels. Arrows point to neurons shown later in detail. Note that in B, the upper left edge mark was shifted slightly away from the barrel border, in order not to cover cell 4b, which is located exactly on the borderline. D: Composite scheme showing the position of the cell-bodies of 35 neurons (with different symbols in the different layers) reconstructed within and close to two barrels (left and middle one in A–C), taken from eight slices in total. Neurons visible in A and B are indicated by their figure numbers. Three-dimensional reconstructions of the two barrels were obtained by determining and superimposing the barrel shapes from the eight consecutive sections. E: The same neurons and barrels after a 90-degree X-rotation, to produce a view from the direction of the pial surface. The eight sections from which these two barrels have been reconstructed are symbolized by dashed horizontal lines. Scale bars = 400 µm.
Fig. 2. Schematic drawings summarizing the main neuron types and their proportion of the whole vasoactive intestinal polypeptide-immunoreactive (VIP-IR) population found in our material, classified according to their somatodendritic morphology (on the left) and axonal arbors (box on the right). Neurons were named bipolar (1) when their perikaryon basically had two poles from which dendrites originated. In most cases, the longitudinal axis of the neuron was vertically oriented. Bitufted neurons were also bipolar, but either had a tuft of dendrites originating directly from the poles of the soma, or from a very short and thick dendritic shaft. Tripolar or modified bipolars differed from bipolars in so far as they had an additional dendritic stem originating from one side of the soma (2). Multipolar neurons had a various number of dendrites originating from different sides of the perikaryon (3). Finally, a number of neurons (i.e., octopus-like), including some only found in infragranular layers, were not considered to belong to any of the neuron types mentioned above, due to the appearance of the perikaryon and the position of the dendrites (4). Concerning axonal arbors, neurons had either vertically ascending or descending axons, rarely both. In layers I–V, axonal arbors were narrow, whereas in deeper layer V and in layer VI, VIP-IR neurons often had wide axonal domains with horizontally extending collaterals.
Fig. 3. Three vasoactive intestinal polypeptide-immunoreactive (VIP-IR) neurons, a bipolar (a), a bitufted (b), and a modified bipolar (c) neuron, from supragranular layers. Axonal arbors are black; arrows point to the axon initial segment. Neurons are vertically oriented and have axons mostly confined to the dendritic domain in the horizontal plane. However, neuron c shows a striking asymmetry toward the barrel. Insets show position of the respective neurons related to barrels. Scale bars = 200 μm; 400 μm in insets.
Fig. 4.  

A: Two weeping willow-like neurons whose perikarya were located in the granular layer, one in a septum (a), the other in a side of the barrel on the right (b). Note the ascending axon (black) with its curved collaterals. Arrows point to the axon initial segment. Arrowheads point to an “erroneous” axon collateral which ramifies into the septum. Inset shows the position of the neurons related to barrels. 

B: Photomontage of neuron a. Note the origin of the axon close to the primary bifurcation of the ascending dendritic stem. Small arrowheads point to the axon; the large arrowhead points to the axon initial segment (AIS). Scale bars = 100 μm in A; 50 μm in B; 400 μm in inset.
both groups had dendrites which were equally restricted in a tangential plane. Therefore, the dendrites of a particular VIP neuron in most cases were spatially restricted to the barrel-related compartment in which this neuron was located. A rarely encountered exception were bipolar neurons in supragranular layers, whose cell bodies were oriented in parallel to the pial surface, and whose dendrites ascended to layer I, giving the neuron a U shape. Although the dendritic arborization was tangentially restricted in all cortical layers, some differences were noticed concerning the layers to which the dendrites of each group extended. Neurons in supragranular and granular layers frequently had upper dendrites which reached layer I, where they bifurcated (Figs. 3, 5). Neurons from lower layer V and layer VI were never found to have dendrites passing over the layer IV–V border (Fig. 6). Dendrites reaching layer IV from below always originated from somata located in upper layer V (Fig. 5).

Concerning axonal arbors, neurons in the first group were seen to have tangentially restricted, vertically oriented arbors with a mean horizontal diameter of about 200 μm (Fig. 7), thus, hardly exceeding the dendritic domain in the tangential direction. In this group, very few neurons were found with a horizontal diameter of the axonal arbor of more than 400 μm. Most of these neurons had axonal arbors whose horizontal diameter was smaller than that of barrels. Accordingly, 85% of all reconstructed neurons in layers I–IV entirely remained in one column, either associated with the barrel or the interbarrel septum in layer IV. Actually, we encountered neurons whose somatodendritic component was located close to the vertical barrel border, and which showed strong asymmetries of the axon tree away from the border and toward the center of a barrel-related column (Fig. 3). However, the axons of other neurons had collaterals that clearly crossed such borders. Nevertheless, they did not extend further than the immediately neighboring barrel column. Therefore, VIP-IR neurons in these layers had a preferentially but not absolutely restricted axonal arbor relative to the compartment in which the perikaryon was located (cf. Fig. 4).

By contrast, neurons of the second group possessed horizontally elongated axonal arbors with a mean tangential diameter of 550 μm. One neuron was even found with horizontal collaterals of more than 800 μm length, which reached across three barrel columns (Fig. 6). In fact, only 46% of these neurons remained in one of the two compartments (barrel- or interbarrel-related columns), whereas 54% of the neurons had axonal arbors not restricted to a barrel column but crossing its borders. Although horizontally extending collaterals seemed to be a feature only found in infragranular layers, it has to be emphasized that it did not belong to all (6 out of 13) reconstructed VIP-IR neurons there. Figure 7 summarizes the numerical distribution of axonal arbors of a particular horizontal diameter within the supragranular, granular and the two parts of infragranular layers.

DISCUSSION

Methodological considerations

We applied immunohistochemical techniques in order to visualize VIP-IR neurons in sections through primary somatosensory cortex. Previous reconstructions of intracellularly filled neurons from rat frontal cortex showed, in comparison to our study, a more complete axonal arbor (Kawaguchi and Kubota, 1996; Porter et al., 1998). However, electrophysiological identification and subsequent filling of neurons only yields a very restricted number of neurons in one animal. In our approach, most if not all VIP-positive neurons were visualized, and the extent of the reconstructions shows that the staining quality was sufficient to achieve a reliable classification based on the visualization of the major axonal trajectories, and basically the entire dendritic tree. However complete and unbiased the whole population of VIP-IR neurons may have been, a selection nonetheless had to occur during the reconstruction of the neurons, because not all stained neurons could be reconstructed.

As we reconstructed the neurons from serial sections not explicitly revealing the laminar architecture of the barrel cortex, it was difficult to determine the exact boundaries between cortical layers. We considered the barrel patches stained by their enhanced cytochrome oxidase activity as a marker of the granular layer, which in fact seems to be a simplification. It has been proposed that barrels delineated by cytochrome oxidase further contain neurons located in deep layer III (Land and Simons, 1985). Therefore, the area and height of layer IV is probably overestimated in our study, whereas the area of the supragranular layers is probably underestimated. Nevertheless, this does not compromise our results, as our observations concerning neurons from supragranular layers and those from the granular layer do not basically differ from each other.

Morphological characteristics of VIP-IR neurons

The population of VIP-IR neurons consisted of mostly bipolar or tripolar (i.e., modified bipolar) neurons with markedly vertical orientation of the dendrites. The dendrites of neurons in supragranular and granular layers often reached the molecular layer, whereas the dendrites of neurons in the infragranular layers generally did not pass to layers II/III. In this respect, VIP-IR neurons in primary somatosensory cortex differ from neurons located in rat motor cortex, intracellularly filled by Porter et al. (1998). Bipolar cells as described by Morrison et al. (1984), characterized by two long dendrites which span long distances in vertical direction with only few bifurcations rather distant from the soma, were rarely encountered, and when present, were mostly in layer V or in supragranular layers. Many VIP-IR neurons in supragranular and granular layers had axons which descended to the granular and infragranular layers similarly to VIP-IR double-bouquet cells reported by Kawaguchi and Kubota (1996) in rat frontal cortex. We also encountered perisomatic baskets consisting of VIP-IR fibers and boutons around the somata of unstained neurons, a phenomenon already reported from rat frontal, cat visual, and monkey prefrontal cortices (Wahle and Meyer, 1989; Kawaguchi and Kubota, 1997; Gabbott and Bacon, 1997). Considerable efforts were made to determine whether these pericellular baskets were derived from a basket cell-like subgroup of VIPergic neurons. We only occasionally found axon collaterals that formed baskets around unstained cell bodies, but these neurons did not seem to comprise a clearly distinguishable entity, as other collaterals of the same neurons behaved differently.
Fig. 5. Neurons with somata located in lower granular and upper infragranular layers. The dendrites of neuron a (bipolar), whose cell body is located in upper layer V, do not reach beyond layer IV. Neuron b (single-tufted), whose cell body is located in the barrel, spans supragranular layers up to layer I with its dendrite. Neuron c (bitufted) in layer IV. Axons are black; arrows point to the axon initial segment. Inset shows position of the three neurons related to barrels. Scale bars = 200 μm.
Fig. 6. Neurons whose somata were located in layer VI. Neurons a (bitufted) and c (bipolar) are characterized by extensive horizontal collateralization of the axon (black). Note the numerous branches ascending from the horizontal collaterals. Neuron b is an octopus-like neuron. All dendrites originate from the upper pole of the soma.

Hardly any axon collaterals could be reconstructed from neurons of this class. Inset shows position of neurons a and c related to barrels. Neuron b was reconstructed from slices not costained with cytochrome oxidase. Scale bars = 200 μm; 400 μm in inset.
Specific manner. Restricted but ramified in both compartments in a less specific manner. Neurons had axons that were not completely restricted but ramified in both compartments in a less specific manner. However, 15% of the reconstructed neurons had axons that were not completely restricted to the same columnar compartment in which the cell body is located. Therefore, the position of the soma should be a subject of future studies.

Cross-correlations of these different classification schemes indicate that there are also layer-specific differences in the morphological properties of neurons within a barrel column, distinguishing them according to initial burst characteristics and colocalization with calretinin and ChAT. They showed that neurochemical differences, such as the presence or lack of colocalization of VIP with these two substances coincided with varying duration of burst firing upon a depolarizing pulse. In this context, it is interesting to note that the colocalization of ChAT and VIP in the barrel cortex of the rat shows layer-specific differences, with a relative lack of neurons colocalizing both substances in infragranular layers than in layers I–IV. In addition to the neurochemical differences, (3) a neurophysiological diversity has been indicated recently. In brief, the population of VIP-IR neurons was shown to consist of regular-spiking (RSNP) and irregular-spiking (IS) or burst-firing cells (Kawaguchi and Kubota, 1997; Cauli et al., 1997). Porter et al. (1998) have reported the IS neurons to consist exclusively of neurons synthesizing VIP, and further subdivided them according to initial burst characteristics and colocalization with calretinin and ChAT. They showed that neurochemical differences, such as the presence or lack of colocalization of VIP with these two substances coincided with varying duration of burst firing upon a depolarizing pulse. In this context, it is interesting to note that the colocalization of ChAT and VIP in the barrel cortex of the rat shows layer-specific differences, with a relative lack of neurons colocalizing both substances in infragranular layers (Bayraktar et al., 1997). This may indicate that there are also layer-specific differences in the physiological properties of VIP-IR neurons in the primary somatosensory cortex. (4) In this study, we show that VIP-IR cells are not only physiologically and neurochemically diverse, but also distinguishable with regard to an association with functional cortical columns. Therefore, cross-correlations of these different classification schemes should be a subject of future studies.

Several publications have pointed at the different functional properties of neurons within a barrel column, depending on the cortical layer in which they are located. Neurons in infragranular layers of the barrel cortex have consistently been reported to contain the widest receptive fields within a barrel column (Chapin, 1986; Armstrong-James and Fox, 1987; Welker et al., 1993; Simons, 1995). The information flow to infragranular layers is both more convergent and divergent and therefore spreads to a more extensive area than in the layers above (Simons, 1978; Chapin, 1986; Armstrong-James and Fox, 1987). The layer-specific variability in the morphology of VIP-IR neurons may be related to this aspect of cortical architecture.

Relationship of the neurons to barrels

A major goal of this study was to examine whether there are indications for a relationship between the location of a VIP-IR neuron inside or outside a barrel-related column and the extent of its processes in relation to these compartments, as has been reported for smooth and spiny stellate cells by some authors (Woolsey et al., 1975; Steffen and van der Loos, 1980; but also see Elston et al., 1997). VIP-IR neurons in layers II-Va possess axonal and dendritic domains of such a small horizontal diameter that it is very likely that both the input and output regions of any of these neurons, like the ones reconstructed, are restricted to the same columnar compartment in which the cell body is located. Therefore, the position of the soma either within a barrel column or the surrounding septal area in granular and supragranular layers proved to be a strong predictor for the area spanned by the axonal arbor. Occasionally the axonal arbors of neurons situated close to the boundaries of barrel columns showed clear asymmetries, oriented toward the compartment where the cell body was located, either within the barrel-related column or in the interbarrel septa. However, 15% of the reconstructed neurons had axons that were not completely restricted but ramified in both compartments in a less specific manner.

Whereas VIP-IR neurons in layers II-Va seem to respect barrel boundaries, the opposite was the case for nearly the half of VIP-IR neurons in lower cortical layers V and VI. The neurons there have rather wide horizontal axonal collaterals, whereas the somatodendritic aspect of these neurons remains narrow and vertically oriented. The differences in the morphology of VIP-IR neurons above and below layer V may be explained in various ways. A possible explanation is that the population of neurons expressing VIP is principally not homogeneous but constituted by different subpopulations with different morphology and associated functions. Indices for such a heterogeneity are: (1) although virtually all neurons co-localize GABA or its synthesizing enzyme glutamic acid decarboxylase (GAD; Kawaguchi and Kubota, 1997; Bayraktar et al., 1997; Porter et al., 1998), only about one-third of all VIP-IR neurons contain choline acetyltransferase (ChAT), the synthesizing enzyme for acetylcholine (Chédotal et al., 1994; Bayraktar et al., 1997); (2) furthermore, partial colocalization of VIP with the calcium-binding protein calretinin and with the peptide cholecystokinin has been reported (Rogers, 1992; Kubota et al., 1994; Kawaguchi and Kubota, 1997). The degree of colocalization of VIP and calretinin has been shown to be layer-specific (Rogers, 1992), as more VIP-IR neurons were found to colocalize calretinin in infragranular layers than in layers I–IV. In addition to the neurochemical differences, (3) a neurophysiological diversity has been indicated recently. In brief, the population of VIP-IR neurons was shown to consist of regular-spiking (RSNP) and irregular-spiking (IS) or burst-firing cells (Kawaguchi and Kubota, 1997; Cauli et al., 1997). Porter et al. (1998) have reported the IS neurons to consist exclusively of neurons synthesizing VIP, and further subdivided them according to initial burst characteristics and colocalization with calretinin and ChAT. They showed that neurochemical differences, such as the presence or lack of colocalization of VIP with these two substances coincided with varying duration of burst firing upon a depolarizing pulse. In this context, it is interesting to note that the colocalization of ChAT and VIP in the barrel cortex of the rat shows layer-specific differences, with a relative lack of neurons colocalizing both substances in infragranular layers (Bayraktar et al., 1997). This may indicate that there are also layer-specific differences in the physiological properties of VIP-IR neurons in the primary somatosensory cortex. (4) In this study, we show that VIP-IR cells are not only physiologically and neurochemically diverse, but also distinguishable with regard to an association with functional cortical columns. Therefore, cross-correlations of these different classification schemes should be a subject of future studies.

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With respect to the vertical orientation of their dendrites, it can be assumed that an as yet undetermined number of afferents distributed across different layers converge on VIP-IR neurons. It is already known that VIP-IR neurons receive a layer-specific pattern of thalamocortical input (Staiger et al., 1996a), which is especially frequent within the barrels and layer III. Due to their dendritic arbors, the main target for these thalamocortical afferents within the VIP-IR neuron population is likely to be the neurons with somata in supragranular layers (see Results). Furthermore, it seems likely that the frequently branching dendrites of supragranular and granular layer VIP-IR neurons are reached by excitatory afferents in layer I. It has been shown that fibers from different neocortical areas pass through and converge in layer I of the rat primary somatosensory cortex, such as fibers from the ipsilateral primary motor and secondary somatosensory cortices, as well as from the contralateral parietal cortex (Caulier et al., 1998) and the nonspecific thalamic nuclei (Lu and Lin, 1993). However, the types of fibers contacting VIP-IR neurons have not been examined to date. Excitatory input from local pyramidal cells to VIP-IR neurons in rat frontal cortex has recently been shown physiologically (Porter et al., 1998) and was morphologically shown to exist (our unpublished observations), at least in the supragranular layers.

As to inhibitory input to VIP-IR neurons, evidence was provided for an extensive inhibitory innervation of VIP-IR neurons by parvalbumin-containing basket cells in rat barrel cortex (Staiger et al., 1997). Parvalbumin-immunoreactive neurons constitute an important class of inhibitory GABAergic interneurons and have the typical features of basket and chandelier cells (Baimbridge et al., 1992; Caulier et al., 1997; Kawaguchi and Kubota, 1997). They have been shown to directly receive specific thalamocortical afferents (Staiger et al., 1996b; Swadlow et al., 1998); in fact, it seems that they are the major targets of thalamocortical afferents among all interneurons immunoreactive for GABA in rat barrel cortex (Staiger et al., 1996b). In contrast to the VIP-IR neurons in layers II–IV, basket cells have a horizontally extended axonal plexus and are thus possible candidates for lateral inhibition in the processing of somatosensory stimuli.

Therefore, it seems that various afferents of both excitatory and inhibitory nature converge on VIP-IR neurons in the different layers. Receiving these afferent inputs, VIP-IR neurons may obtain a summarizing impression of the actual state of activity in the respective columnar area. In turn, they could perform a coupling of the state-dependent fine-tuning of the local neuronal activity (by GABA-mediated inhibition or the release of the neuro-modulatory substances VIP and acetylcholine) with the metabolic demands (by regulating blood flow and glycolysis). In this respect, ascending axonal arbors of VIP-IR neurons in layer IV, such as the weeping willow-like cells, may be integral parts of feed-forward mechanisms primarily driven by thalamocortical input and targeting the supragranular layers. VIP-IR axons show a layer-specific association to the barrel-related compartment in which the soma is located. In layers II–V, this association is rather tight, resulting in vertically oriented, narrow axonal arbors. In layers VB and VI, it is often lost, due to an extensive horizontal collateralization of the axons. Thus, VIP neurons seem to influence the cellular activity and metabolism in either a restricted or wider ensemble of neurons, depending on their location in the upper or lower part of the cortex, respectively. We propose that this parallels to some extent the functional principle effective in these two divisions of cortex, the upper being more extractive and the lower being more integrative (Simons, 1978; Armstrong-James and Fox, 1987). Neurons in infragranular layers have large but weak receptive fields and respond to the stimulation of more whiskers than do neurons in the layers above (Armstrong-James and Fox, 1987). As neurons in the infragranular layers do not seem to maintain a somatotopy as strict as the neurons above, it can be assumed that VIP-IR cells innervating them will also span a less well-defined but extensive area of cortical tissue, in order to appropriately adjust the neuronal excitability and metabolism in this region.

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