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Map of the Synapses Onto Layer 4 Basket Cells of the Primary Visual Cortex of the Cat

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ABSTRACT

The pattern of excitatory and inhibitory inputs to the inhibitory neurons is largely unknown. We have set out to quantify the major excitatory and inhibitory inputs to layer 4 basket cells from the primary visual cortex of the cat. The synapses formed with the soma, and proximal and distal dendrites, were examined at the light and electron microscopic levels in four basket cells, recorded in vivo and filled with horseradish peroxidase. The major afferents of layer 4 have been well characterised, both at the light and electron microscopic levels. The sizes of the synaptic boutons of the major excitatory inputs to layer 4 from the thalamic relay cells, spiny stellate cells, and layer 6 pyramidal neurons are statistically different. Their distributions were compared to those of the boutons forming asymmetric contacts onto the basket cells, which were assumed to be provided by the same set of excitatory afferents. The best-fit results showed that about equal numbers of synapses were provided by the layer 6 pyramids (43%) and the spiny stellates (44%), whereas the thalamic afferents contributed only 13%. A similar analysis on the symmetric synaptic input to the basket cells indicated that as much as 79% of the symmetric synapses could have originated from other layer 4 basket cells. Thalamic and spiny stellate synapses were preferentially located on the soma and proximal dendrites, regions that also had 76% of all the symmetric contacts. J. Comp. Neurol. 380: 230–242, 1997.

Indexing terms: serial EM; morphometry; bouton size distributions; synaptic quantification; smooth cells

The γ-aminobutyric acid containing (GABAergic) neurons play a key role in cortical circuits (Tsumoto et al., 1979; Berardi et al., 1980; Sillito et al., 1980; Douglas et al., 1989; Berman et al., 1991; Pei et al., 1994). It is not surprising then that the output of these neurons has been intensively studied, in terms of both the three-dimensional distribution of their axons and their synaptic connections to other neurons (Kisvárday et al., 1985; Gabbott et al., 1988; Kisvárday, 1992). These studies have shown that the subclasses of neurons not only have characteristic axonal arborizations, but also form synapses at different positions on their main target neurons, which are the pyramidal and spiny stellate cells of the cortex.

It is not the purpose of the present study to resolve the many issues surrounding the current debate about the precise role of inhibitory neurons of layer 4 (Das, 1996; Ferster et al., 1996; Hubel, 1996; Vidyasager et al., 1996). However, any functional model of layer 4 requires basic information as to the structure of the circuits of layer 4. We have undertaken this study with the purpose of quantifying the sources of excitatory and inhibitory inputs to basket neurons of layer 4. We have selected the basket cells of layer 4 for study because they appear to be the major type of inhibitory neuron found in layer 4 of the cat's visual cortex. In vivo they have the small receptive field sizes typical of layer 4 (Hubel and Wiesel, 1962; Gilbert, 1977), and most are simple cells (Gilbert and Wiesel, 1979; Martin et al., 1983; Gabbott et al., 1988). The very localised arrangement of their axonal arborisation indicates that most of the neurons they inhibit are their neighbours, which are predominantly other simple cells of layer 4.

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Whereas the output of these neurons has been well specified anatomically, their input has not (see White, 1989; Kisvárday, 1992). Most studies have used serial electron microscopic (EM) reconstructions in combination with tracing techniques to show that smooth or GABAergic neurons in layer 4 were a major target of the thalamic projection to layer 4 (Davis and Sterling, 1979; Hamos et al., 1983; McGuire et al., 1984; Freund et al., 1985; Einstein et al., 1987, in the cat visual cortex; and Keller and White, 1987, in the mouse barrel cortex). McGuire et al. (1984) used similar methods to show that the recurrent collaterals of the layer 6 pyramidal cells provided a strong input to the smooth neurons of layer 4. Although it is likely that layer 4 basket cells were among the targets of these recurrent collaterals, the detailed morphology of the postsynaptic neurons could of course not be determined. Direct evidence that layer 4 basket cells were monosynaptically excited by thalamic afferents was obtained by electrophysiological methods in combination with intracellular staining of the basket cells (Martin et al., 1983; Kisvárday et al., 1985; Gabbott et al., 1988).

In this study, we mapped the distribution of synapses formed with the dendrites of layer 4 basket cells and established their likely origin by matching the morphology of the synapses and presynaptic boutons with the profile of axons that are known to form boutons in layer 4, i.e., thalamic afferents, and axons of cortical neurons including spiny stellates, layer 6 pyramids, and basket cells. By this technique, we were able to estimate quantitatively the proportion of synapses contributed by the different afferent inputs to the basket cells. The results showed that the basket cells received a high density of synapses, particularly on the proximal dendrites. Asymmetric synapses originating from cortical spiny neurons contributed the major part of the total synaptic input. There was also a significant minority of synapses formed with thalamic afferents, indicating that these layer 4 basket cells participate in both feed-forward and recurrent inhibitory circuits. Our examination of symmetric synapses indicated a range of sources, with a major contribution from other layer 4 basket cells.

METHODS

Physiology

Adults cats were prepared for physiological recording from neurons of the visual cortex, area 17 (Martin and Whitteridge, 1984; Douglas et al., 1988). Anaesthesia was induced with a gas mixture of 1–3% halothane (May and Baker) in oxygen/nitrous oxide (50%/50%). Surgery was performed under gas anaesthesia supplemented with Saffan (alphaxolone/alphadolone, Glaxovet). Pairs of tungsten-stimulating electrodes were placed at the optic chiasm (site OX) and the optic radiations above the dorsal lateral geniculate nucleus (dLGN, site OR1), and in the white matter immediately beneath the recording position (site OR2). After surgery the cats were given an intravenous infusion of the muscle relaxants gallamine triethiodide (80 mg induction dose, 13 mg kg^{-1} hr^{-1} thereafter) (Sigma, Poole, U.K.) and Tubocurarine (Wellcome, U.K.); they were then artificially ventilated with a mixture of oxygen/nitrous oxide (30%/70%). Anaesthesia was maintained by intravenous Saffan or sodium pentobarbital (Sagatal, May and Baker, 2–3 mg kg^{-1} hr^{-1}). EEG, blood pressure, heart rate, end-tidal CO_2, and rectal temperature were monitored continuously. These experiments were carried out under Programme and Personal licences granted by the Home Office Animals (Scientific Procedures, U.K.) Act of 1986. They followed the Codes of Practice and Protocols established by the Medical Research Council and by the University of Oxford Veterinary Officers.

Glass recording electrodes were filled with 4% horseradish peroxidase (HRP; Boehringer Grade 1) in 0.2 M KCl and 0.05 M Tris (Sigma), pH 7.9. The tip of the recording electrode was bevelled to about 80–100 MOhms. The receptive field and latency to electrical stimulation of each cell was determined prior to intracellular recording and filling with HRP. Neurons were classified as monosynaptically activated if they responded with latencies of 1.7 ms or less after stimulation at site OR2, or 2.0 ms or less from site OR1 (see Martin and Whitteridge, 1984).

Histology

At the end of the physiological recording the cat was given an overdose of sodium pentobarbital and perfused transcardially with a fixative solution of 1% paraformaldehyde and 2.5% gluterladehyde in 0.1 M phosphate buffer (pH 7.4). The block of tissue containing the intracellularly filled cell was serially sectioned in the coronal plane at a thickness of 80 μm by using an Oxford Vibratome. The sections were incubated by using the procedure of Hanker et al. (1977) with nickel/cobalt intensification (Adams, 1981). Sections containing HRP-filled processes were treated with 1% osmium tetroxide in 0.1 M phosphate buffer, dehydrated with alcohols and propylene oxide, and flat-embedded in Durcupan ACM (Fluka) resin for light microscopy (LM). Uranyl acetate (1%) was included in the 70% alcohol during dehydration in order to enhance contrast in the electron microscope (EM).

GABA immunoreactivity was tested on serial sections mounted on nickel grids by using the postembedding immunogold method of Somogyi and Hodgson (1985). The resin was etched by using 1% periodic acid (20 minutes), and osmium was removed by using 2% sodium periodate (10 minutes). All reactions were carried out on drops in a petri dish. Ovalbumin was used as a general blocking agent (1%). The washing steps used Tris buffered saline (TBS at pH 7.4) and normal goat serum (1%) dissolved in TBS, prior to and for dilution (1:2000) of the primary antiserum to GABA. The sections were incubated in primary antiserum overnight at 4°C. The secondary antibody, goat anti-rabbit IgG conjugated to 15 nm colloidal gold, was dissolved in 1% bovine serum albumen in Tris buffer. The sections were incubated in secondary antibody (1:20) for two hours at room temperature. After the final washing steps, the sections were treated with a saturated aqueous solution of uranyl acetate (40 minutes), followed by lead citrate (4 minutes) to enhance the final contrast.

Reconstruction at LM and EM

Cells were reconstructed in 2-D by using a light microscope and drawing tube attachment magnified to ×600, and in 3-D by using an in-house 3-D reconstruction system (Traka). Selected portions of the axons and dendrites were photographed in the light microscope before being re-embedded and serially sectioned at 70-nm thickness. The serial ultrathin sections were collected on Pioloform-coated single-slot copper or nickel grids and photographed

at 16,900 times. The serial electron micrographs were then digitised in order to reconstruct the boutons and the postsynaptic targets. The digitised profiles from individual sections were merged as a series by using customised computer software (Trakem, supported by Calcomp bitpad, Research Machines VX486). The merged structures consisted of a series of superimposed wire profiles that could be rotated to give alternative views of the arrangements of synaptic inputs. A semi-automated system was used to measure the lengths and areas of digitised profiles, e.g., dendritic shafts, boutons, and pre- and postsynaptic junctions. From these reconstructions we estimated the density of synapses per unit length and measured the longest synaptic length and the cross-sectional area of the presynaptic bouton associated with that synaptic profile. Such data have been used for the procedure of "template matching" (Ahmed et al., 1994). These measures were taken for all complete synapses; and in conjunction with the synapse type (asymmetric or symmetric), these measures enabled us to estimate the relative contribution of presynaptic afferents of layer 4 to our sample of basket cells.

Statistical fitting procedure

The statistical fitting procedure is similar to that used in a previous study of layer 4 spiny cells, their boutons and dendrites (Anderson et al., 1994a,b; Ahmed et al., 1994). We have used data from these studies that described the distributions of boutons formed by thalamic afferents, spiny stellates, layer 6 pyramidal cells, and basket cells of layer 4. New values were added to the data from spiny stellate cells and thalamic afferents. In the original fitting procedure we used the median and the standard deviation for each presynaptic distribution to fit to the data derived from the boutons that formed synapses with the spiny stellate dendrite (Ahmed et al., 1994). However, with new data these bouton distributions appeared more distinctly skewed (see Fig. 5A), so in the present study we used a log-normal function to find the best statistical fit to these distributions. We then used the derived mean and variance of these log-normal distributions in linear combination to find the "best fit" to the bouton cross-sectional data from the basket cell soma, and proximal and distal dendrites. This protocol was based on the non-linear, least-square estimation procedure of minimising the sum of squared deviations (Statistica, StatSoft, Inc., Tulsa, OK) to fit the three distributions from the three sources of asymmetric synapses collectively to the distribution from the basket cells. The final fit represented the statistically best-fit situation (minimised χ^2). A similar procedure was used to fit the bouton distribution from the layer 4 basket cell axon to the presynaptic symmetric bouton distribution obtained from the serially sectioned soma and dendrites of basket cells.

RESULTS

Physiology and light microscopy

Four basket neurons from layer 4 were recovered from in vivo recording from area 17 of the cat's visual cortex. The soma of these cells was located in layer 4A at the top of the postcentral gyrus between stereotaxic coordinates posterior 3–6 mm. The receptive fields were located within 10 degrees of the *area centralis*. Two of these cells had simple type fields: an S_1 (ocular dominance [OD] group 7, directionally biased, and showed length summation); and an S_2 (OD group 3). One had a complex, length-summating, (Cstd)-receptive field, was OD group 7, and was directionally biased. The electrical stimulation electrodes activated this cell from OX, OR1, and OR2 with latencies, respectively, of 4 ms, 2.2 ms, and 1.4 ms. The OX-OR1 difference was intermediate between Y- and X-type conduction rates. The fourth cell had a non-oriented concentric receptive field with an off-centre and on-surround. The cell was monocular (OD group 7); it had a latency of 3.6 ms to OX stimulation, which indicates monosynaptic activation from the dLGN, probably by the fast-conducting Y-pathway. Functionally, therefore, these cells were representative of the major classes of receptive field types found within layer 4.

Each neuron had a multipolar arrangement of dendrites that were characteristically smooth, varicose, and branched, and were restricted to layer 4. Figure 1 illustrates a typical example of the four cells, and of layer 4 basket cells in general. The major and minor somal diameters for this neuron were, respectively, 16 µm and 10.2 µm. In general, four to six primary dendrites emerged from the somas of these cells with the first branch point at a mean length of 16 µm (s.d. = $\pm$10.3 µm, n = 13). In two cells a primary dendrite remained unbranched throughout its length. Each cell possessed a well-filled axon that formed a dense axon plexus largely confined to layer 4. In previous studies we confirmed with the EM that boutons which are seen to form contacts with somata or dendritic shafts under the light microscope do form synapses with those targets. In addition they form synapses with dendritic spines (Freund et al., 1985; Kisvárday et al., 1985). The axon typically formed many short collaterals with distinctively large *en passant* boutons, which gave the axonal arbors of the layer 4 basket cells their dense, local, and clustered appearance. The axon collaterals extended both into supra- and infragranular layers. In layer 3, the axon collaterals were diffuse and extended to layer 2. In infragranular layers, a single axon collateral, unbranched, extended into and terminated within either layer 5 or layer 6.

Electron microscopy: Quantification of input to dendrites and soma

Dendrites that lay roughly parallel to the sectioned surface were selected for resectioning, because this reduced the total number of serial sections required for the EM reconstructions. A greater length of dendrite can be examined using fewer sections than would be the case if the dendrite were approached from a more orthogonal plane. In one neuron, the synapses could be mapped along the dendrite, but the ultrastructural quality of their presynaptic boutons was too poor to permit secure measurements of their areas. Of the remaining three cells, one was lightly stained, which made the internal structure easily visible, especially over the proximal portions of the dendrites and soma (Figs. 1, 2). This neuron was used to examine the input to the soma.

GABAergic neurons form symmetric synapses; therefore, as an additional check on our morphological classification of synapses, a sample of boutons that formed synapses with the basket cell dendrite were tested for immunoreactivity for GABA. Figures 2A and 2B show electron micrographs of ultrathin sections of a basket cell dendrite that formed synapses with three boutons. Two of the synapses were asymmetric and one was symmetric. After removing the HRP the section was tested for GABA immunoreactiv-

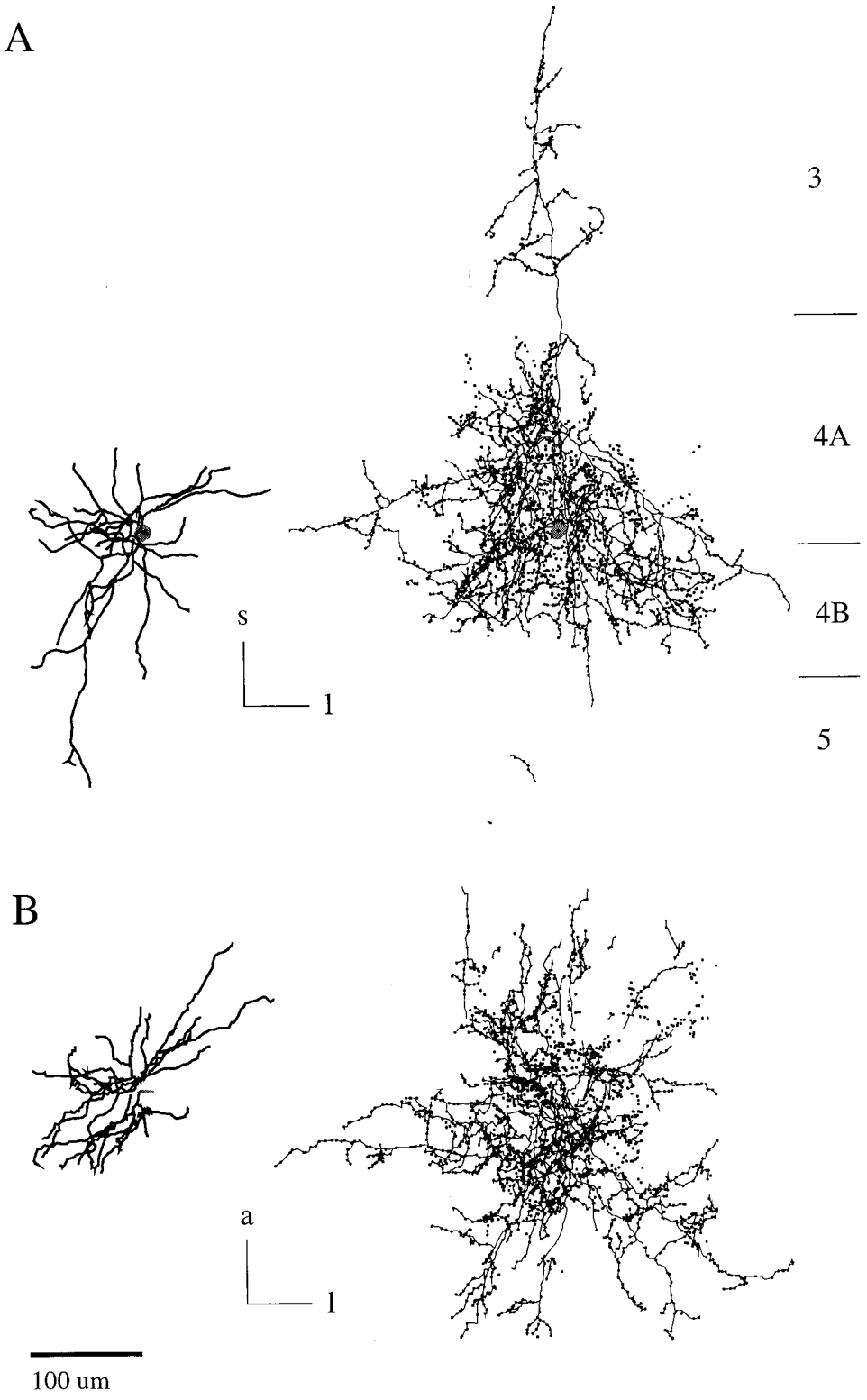

Fig. 1. Light microscopic 3-D reconstruction of a lightly filled layer 4 basket cell used in this study. The dendrites and axon have been separated for ease of viewing. **A:** Coronal view of dendrites (left) and axon (right). The soma is represented by a small hatched polygon. Cortical laminae 3, 4A, 4B, and 5 are shown to the right of the axon. The orientation bars indicate superior (s) and lateral (l). **B:** Rotation of the cell through 90° to provide the view from the pial surface. The orientation bars indicate anterior (a) and lateral (l). Scale bar = 100 μm.

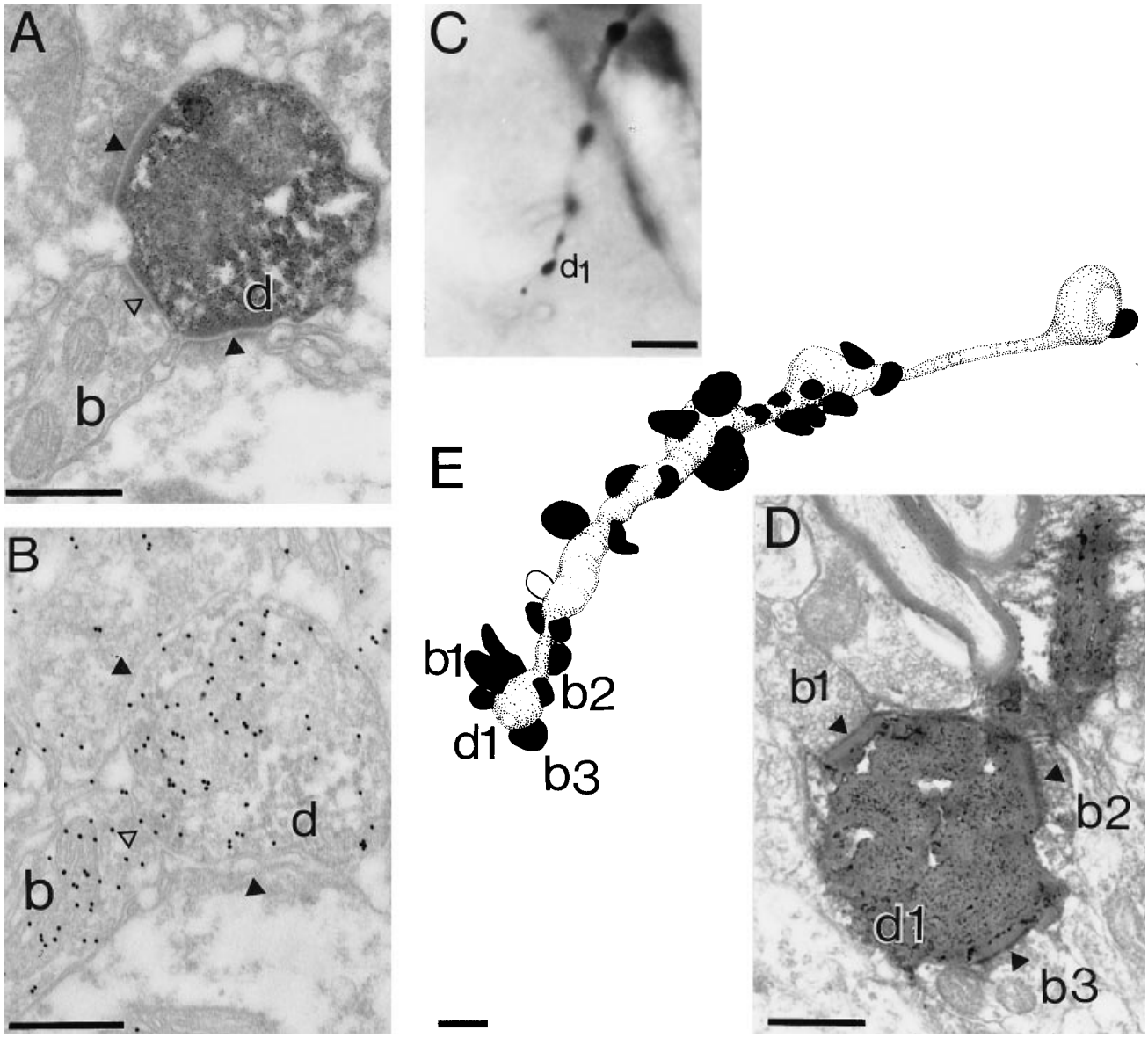

Fig. 2. Light and electron micrographs and a reconstruction of layer 4 basket cell dendrites filled with horseradish peroxidase (HRP). **A:** HRP-filled profile through proximal dendrite of cell shown in Figure 1. The electron-dense dendrite (d) contains two mitochondria and forms synapses with three unidentified boutons. Two of the boutons form asymmetric synapses (filled arrowheads); the third bouton (b) forms a symmetric synapse (open arrowhead). **B:** An almost adjacent section to that shown in A that has been immunostained for γ-aminobutyric acid (GABA). The accumulation of gold particles over the layer 4 basket cell dendrite (d) and the bouton (b) forming the symmetric synapse indicate that both are GABA-positive. C, D, and E are taken from the distal portion of a dendrite of a second layer 4 basket cell. **C:** Light micrograph of a distal tip of a dendrite, d1 is the penultimate varicosity. **D:** Electron micrograph of a section through d1. Three boutons (b1, b2, b3) form asymmetric synapses (filled arrowheads) with the dendrite, d1. **E:** Reconstruction of dendrite shown in C and D, showing boutons that make synapses with the dendrite. The positions of boutons b1, b2, and b3 are indicated. Boutons forming asymmetric synapses are black, and those forming symmetric synapses are white. The dendrite has been rotated slightly to optimise the view of the boutons. Scale bars = 0.5 μm in A, B, D, 5 μm in C, 1 μm in E.

ity. Both the bouton that formed the symmetric synapse and the basket cell dendrite had a higher density of gold particles over them than did both the boutons that formed asymmetric synapses and the surrounding neuropil. Thus, both the basket cell dendrite and the bouton forming the symmetric synapse were GABA-immunopositive. This result supported the morphological classification of synapses as either symmetric or asymmetric, with the GABAergic boutons forming symmetric synapses (Freund et al., 1983; Somogyi and Soltész, 1986).

Taken altogether, the regions sampled from the three neurons provided a total of 48.7 μm of proximal dendrites and 213.2 μm of distal dendrites, and one somal surface area of 46.5 μm^2. The ultrastructural quality and staining of the soma was such that densely packed mitochondria and other organelles, as well as the presynaptic boutons, were visible in the EM. The nucleus occupied the central 30% of the soma and had a small, shallow invagination. We examined a total of 423 synapses made with our selected dendrites and soma. The results of the analysis of these

TABLE 1. Sample Statistics for Layer 4 Basket Cells Obtained From Light Microscopy (LM) and Electron Microscopy (EM) Reconstructions of Dendrites of 3 Basket Cells and Soma of One Basket Cell[1]

	Soma	Proximal dendrite	Distal dendrite	Total
EM serial reconstruction				
Number	1	4	6	
Total length/area	46.5 μm^2	48.7 μm	213.2 μm	
Synaptic boutons forming:				
Asymmetric synapses	24	89	266	379
Symmetric synapses	4	23	6	33
Unclassified	4	7		11
Total	32	119	272	423
Synaptic density:				
Asymmetric synapses	0.52 μm^{-2}	1.94 μm^{-1}	1.25 μm^{-1}	
Symmetric synapses	0.09 μm^{-2}	0.47 μm^{-1}	0.03 μm^{-1}	

[1]Synaptic densities averaged for total length of dendrite sampled.

boutons are summarised in Table 1. Most symmetric synapses (23/33) were formed with the proximal dendrites. This was also the region most densely innervated by boutons that formed asymmetric synapses. Only six symmetric synapses were formed on distal regions of dendrite. In two cases (two different cells), a symmetric synapse occurred on the most distal parts of the dendrite, within 10 μm of the tip (Figs. 2E, 4).

The asymmetric synapses formed 90% of all synaptic contacts onto the soma and dendrites of layer 4 basket cells. However, the serial reconstructions shown in Figures 2–4 show clearly that the synapses formed with the distal dendrites are not homogeneously distributed. Instead they occur in clusters. These clusters are located on the beads of the dendrites, which were filled with mitochondria (Fig. 2A,D). The positions of the beads are indicated by the brackets in the summary diagram of Figure 3. The inter-bead segments receive few or no synapses (Figs. 2E, 3). The presynaptic boutons that formed the clusters were heterogeneous in size. It is also clear from the summary diagram of Figure 3 that the density of synapses changes greatly from proximal to distal. Analysis of bouton densities along a single dendrite from the soma to 145 μm distally showed that the density declined after 30–40 μm from the soma (Fig. 3). Examination of synaptic densities in three other proximal dendrites (<35 μm from soma) corroborated this finding. We thus used this distance of 35 μm as the point at which to make the cut-off of proximal from distal dendrites (Table 1; Figs. 3, 4). We also examined the synaptic input to the soma of one of the basket cells, which had an estimated somal surface area of 506 μm^2 and a mean synaptic density of 70 synapses 100 μm^{-2}. The majority of synapses on the soma were asymmetric (75%, Table 1).

The mean dendritic length of the neurons was 3243 μm (n = 3), with the total proximal length of 415 μm and distal length of 2828 μm (Table 2). Based on these measurements and the derived synaptic densities (Table 1), we calculated that on average a layer 4 basket cell receives a total of 5026 synapses of which 93% are asymmetric and 7% symmetric. Of the mean number of asymmetric synapses, 25% were located on the soma and proximal dendrites. The differential distribution of symmetric synapses

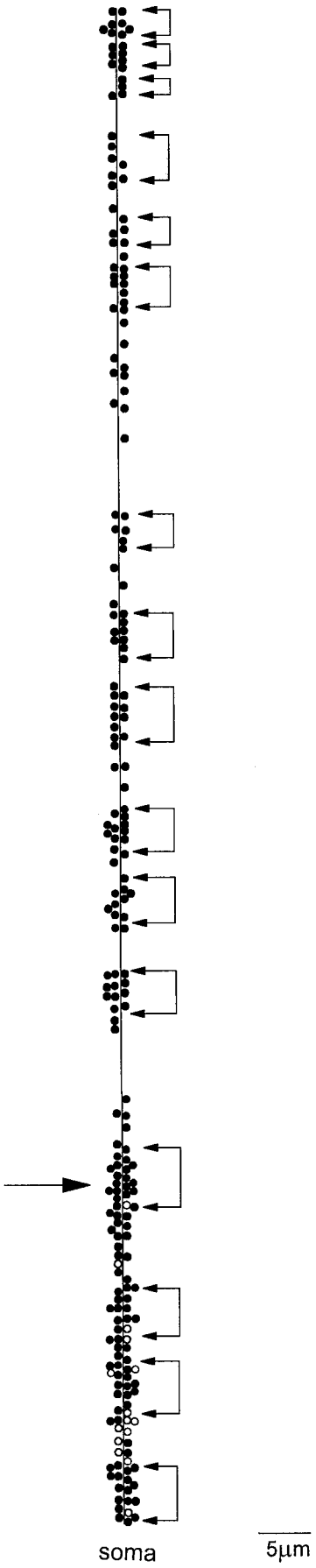

Fig. 3. Schematic summary diagram of a serially sectioned dendrite taken from the neuron shown in Figure 1. Solid line represents the HRP-filled dendrite; asymmetric synapses are represented by filled circles, symmetric synapses by open circles. Branch point indicated by large arrow. Varicose swellings of dendrite are indicated by small connected arrowheads. Scale bar = 5 μm.

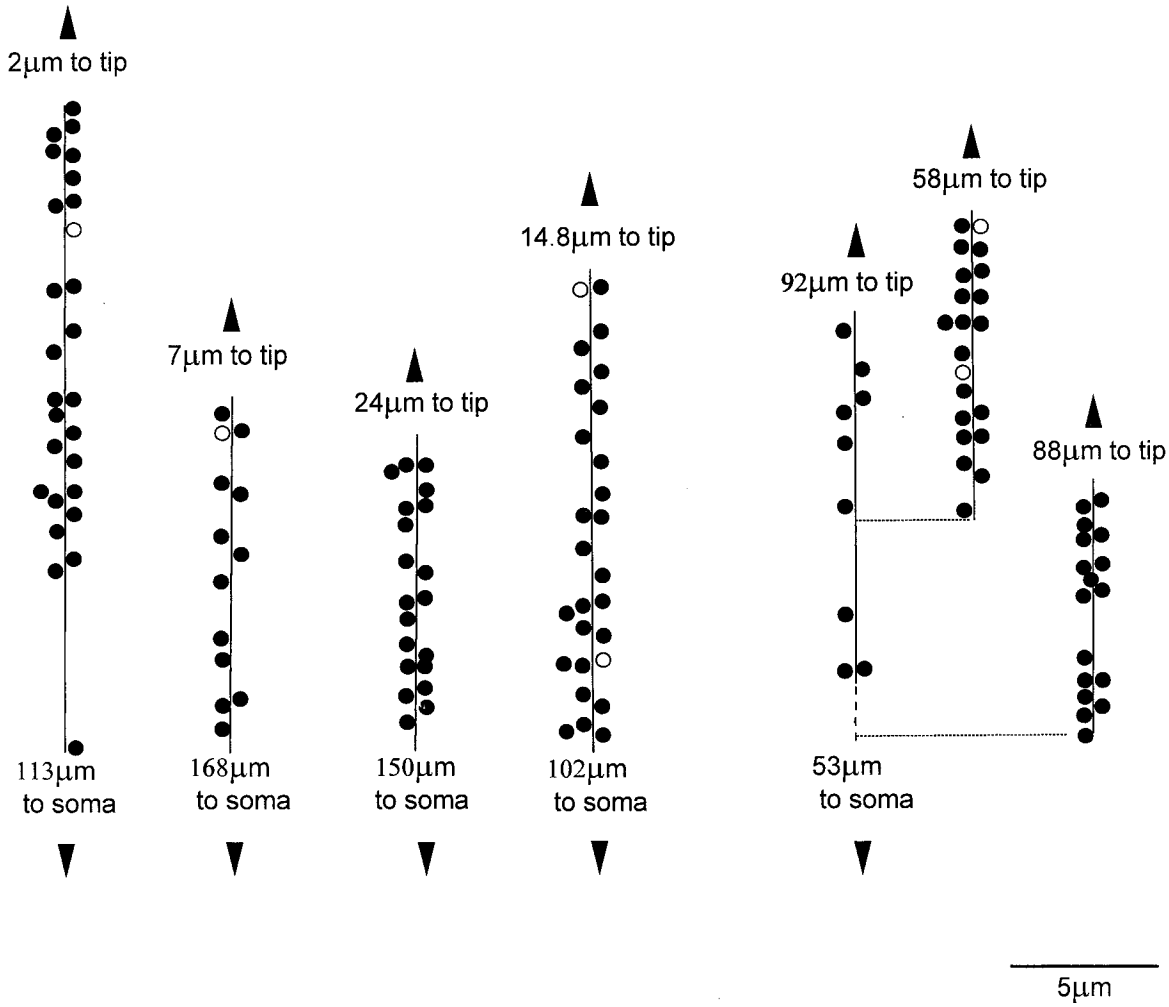

Fig. 4. Schematic summary diagrams of the serially sectioned dendrites. Solid line represents the HRP-filled dendrite; asymmetric synapses are represented by filled circles, symmetric synapses by open circles. Dotted horizontal lines are branch points. Dashed vertical lines are unexamined sections of dendrite. Distance to tip and soma is noted between dendrite and arrowhead. Scale bar = 5 μm.

TABLE 2. Dimensions and Total Numbers of Synapses for a "Typical" Layer 4 Basket Cell Estimated From 3-D LM Reconstructions and Data of Table 1

	Soma	Proximal dendrite	Distal dendrite	Total
Layer 4 basket cell				
Area	506 μm²			
Mean length		415 μm	2828 μm	3243 μm
Synaptic numbers/cell:				
Asymmetric synapses	305	855	3528	4688
Symmetric synapses	50	208	80	338
Total	355	1063	3608	5026

meant that the proximal dendritic region accounted for 62% of all inhibitory synapses on an equivalent of 13% of the total dendritic length. The proximal dendrite had a significantly higher density of asymmetric synapses; and the distribution of presynaptic bouton cross-sectional areas (n = 89, mean = 0.46 μm²) was significantly larger ($P < 0.001$) than on the distal dendrites (n = 266, mean = 0.32 μm²).

Source of presynaptic boutons

Under the light microscope we had previously observed that the size of HRP-labelled axonal boutons varied according to their source. The thalamic afferents, either X- or Y-type, and the basket cells had the largest boutons. The layer 6 pyramidal cell axons had the smallest boutons, while the spiny stellate axons had intermediate-sized boutons. Subsequent EM examination indicated that the cross-sectional areas of these boutons were significantly different and thus, together with the type of synapse, could be used as a means of identifying the source of unstained boutons in the electron micrographs of the basket cell dendrites. Specifically, we used a strategy previously employed in the analysis of boutons in synaptic contact with identified dendrites (Ahmed et al., 1994) to determine the composition of the distribution of boutons presynaptic to basket cells. Anatomical studies have identified three major excitatory inputs to layer 4: relay cells of the dLGN, layer 6 pyramidal cells, and spiny stellates. The major source of inhibition is thought to be from the layer 4 basket

cells (Martin et al., 1984; Kisvárday et al., 1985; Kisvárday, 1992). In previous studies we recorded from layer 4 basket cells and filled them intracellularly with horseradish peroxidase. Boutons from the axons of these identified neurons were serially sectioned and examined in the EM. For the present study we used serial electron micrographs from previous studies supplemented with new material to obtain the data analysed here (Dehay et al., 1991; Anderson et al., 1994a,b). Axons from two thalamic afferents, six spiny stellates, five layer 6 pyramids, and two layer 4 basket cells provided the sampled boutons. The excitatory afferents all formed type 1 or asymmetric synapses with their targets, whereas the boutons of the basket cell axons formed type 2 symmetric synapses. Their targets (mainly dendritic shafts or spines) were classified according to conventional criteria (Peters et al., 1991).

With the analysis of additional material, we increased the sample size of the spiny stellate and thalamic bouton numbers from our earlier sample used to determine the source of synapses formed with spiny stellate dendrites (Ahmed et al., 1994). The distributions for the bouton cross-sectional areas obtained from these axons from the spiny stellates (n = 60), the layer 6 pyramids (n = 38), and the thalamic afferents (n = 40) within layer 4 are shown in Figure 5A. These distributions were skewed and were best fit statistically by a log-normal distribution (R-squared = >0.9, continuous curve in Fig. 5A). In our previous analysis of the spiny stellate cells we used normal distributions (Ahmed et al., 1994), but the log-normal distribution gave closer fits to these new data. For each of these distributions, the mean (μm^2) and variance values were 0.18, 0.27 for layer 6 pyramidal cells; 0.32, 0.34 for spiny stellate cells; and 0.76, 0.08 for the thalamic afferents. The distributions of bouton sizes from the layer 4 basket cell axons were, similarly, also fitted by log-normal distributions (Fig. 5B). The distributions of the identified presynaptic boutons were fitted separately to the data from the soma and the proximal and distal dendrites of the basket cells. The cross-sectional areas of boutons forming asymmetric synapses with basket cells are shown in Figure 6A and B for the somal (n = 24) and proximal (n = 89) and distal (n = 266) dendritic regions. Figure 6A shows the raw data. Figure 6B shows the data normalised for a 100 µm length of dendrite or soma perimeter. These normalised data were used for the "template matching" of the identified bouton distributions to the unlabelled boutons that formed synapses with the basket cell dendrite. The protocol for the statistical fitting was based on the least-squared estimation procedure (Statistica, StatSoft, Inc.) outlined in Methods.

The three distributions for the identified boutons that formed asymmetric synapses (Fig. 5A) were fitted to the data of Figure 6B. The results are illustrated in Figure 7. The final fit represented the best fit statistically (minimised χ^2). For the somal, proximal, and distal dendritic data, respectively, 81%, 92%, and 99% of the variance could be accounted for by the fitted distributions. The somal data were best fit if only the distributions from spiny stellates and thalamic afferents were used. The distal dendritic data were extremely well fitted by a combination of the three excitatory inputs. In the proximal case, although the three excitatory distributions in combination accounted for 92% of the inputs, there was a clear peak in our data (see Fig. 6, proximal dendrite) with bouton areas around 0.65 μm^2, which could not be fitted by a combination of these three excitatory inputs. For the distal dendrite a small secondary peak was also seen around bouton areas of 0.55 μm^2 (Fig. 6, distal dendrite). The boutons that formed asymmetric synapses with the soma and proximal dendrites were weighted toward larger cross-sectional areas (see Fig. 6A). Based on these fits, the calculated contribution of these three excitatory sources to the basket cell (synapses per cell and %) are summarised in Figure 7.

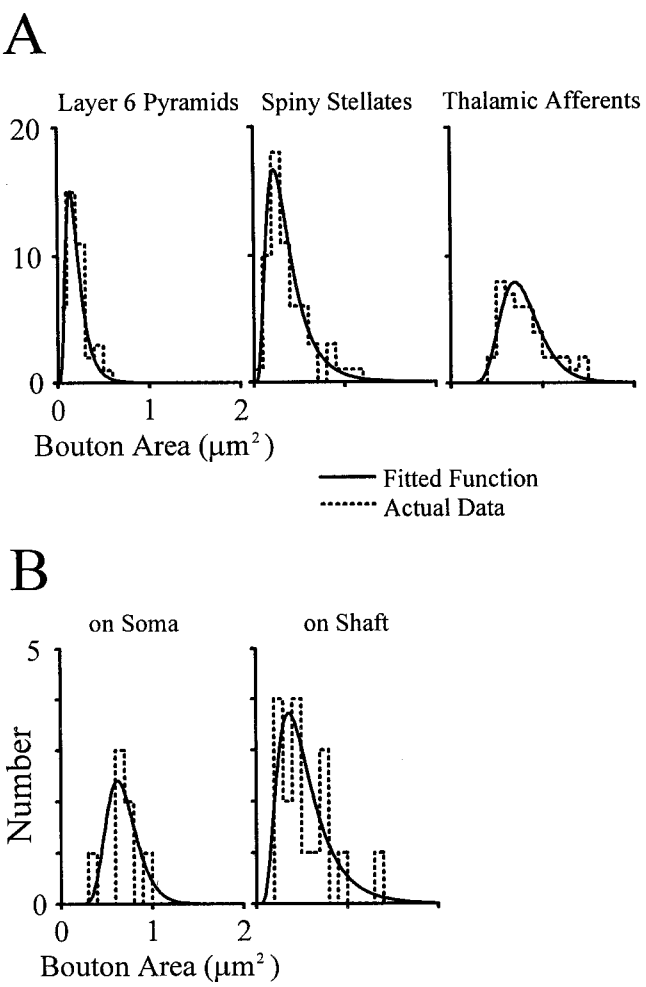

Fig. 5. Distribution within layer 4 of the cross-sectional areas of boutons. **A:** Bouton size distribution (dashed line) of axons from identified layer 6 pyramids, spiny stellates and X and Y type thalamic afferents. **B:** Bouton size distribution for layer 4 basket cell axons (dashed lines). The continuous curve in each figure is the respective log-normal distribution that best fits these data.

We have followed a similar procedure to fit the data from the symmetric synapses formed with the basket cell dendrites (Fig. 8). First, log-normal distributions were fitted to the measured cross-sectional areas of the boutons from identified axons of layer 4 basket cells (Fig. 5B). Then, for the identified basket cell boutons that formed synapses with somata and dendritic shafts, the distributions of bouton cross-sectional areas (continuous curves of Fig. 5B) from identified basket cell axons indicated that the boutons forming synapses with somata had larger cross-sectional areas (mean 0.66 μm^2, variance 0.7) than for the boutons forming synapses with the dendritic shafts (mean 0.47 μm^2, variance 0.27).

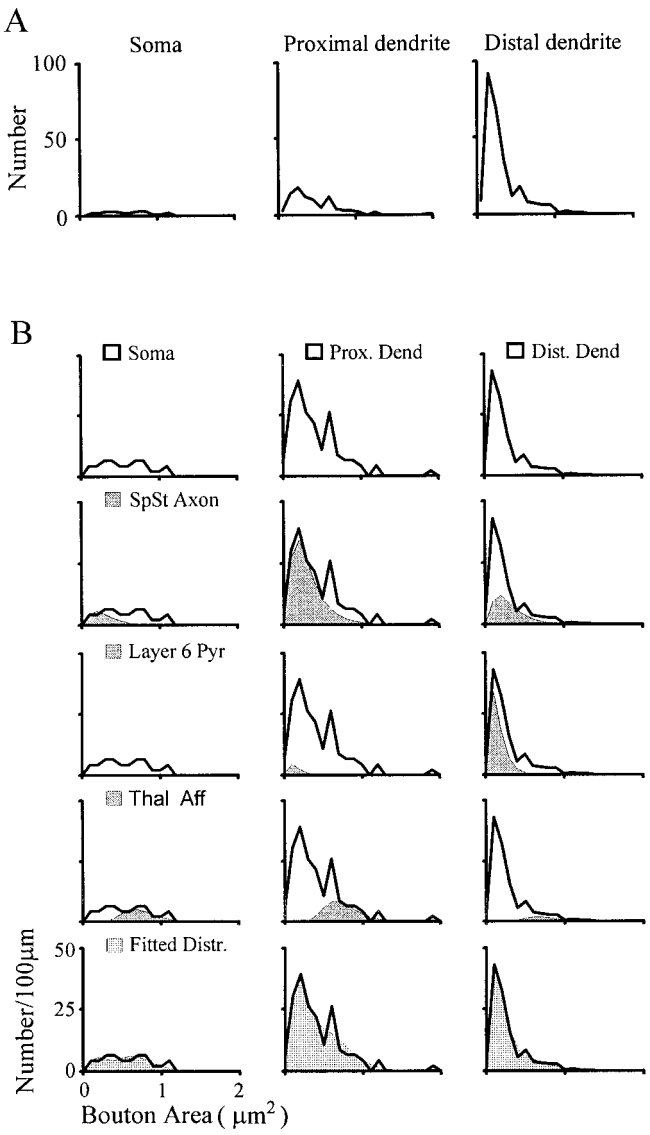

Fig. 6. **A:** Distributions of asymmetric synapses onto different regions of basket cells of layer 4 in cat visual cortex—the raw data. **B:** Asymmetric synaptic input distributions to the soma, and the proximal (Prox. Dend.) and distal (Dist. Dend.) dendrite of basket cells of layer 4. The first row of figures are the normalised distributions from Figure 6A. The filled distributions below are the bouton data that in combination contributed to the overall asymmetric synaptic input onto the soma and dendrites of the layer 4 basket cells from the spiny stellate (SpSt Axon), layer 6 pyramids (Layer 6 Pyr), and thalamic afferents (Thal Aff, see Fig. 5A). The relative contribution of each of these excitatory sources was determined by a least-square statistical fitting procedure. The bottom figures show the combined final fit (correlation coefficient, R = >0.90).

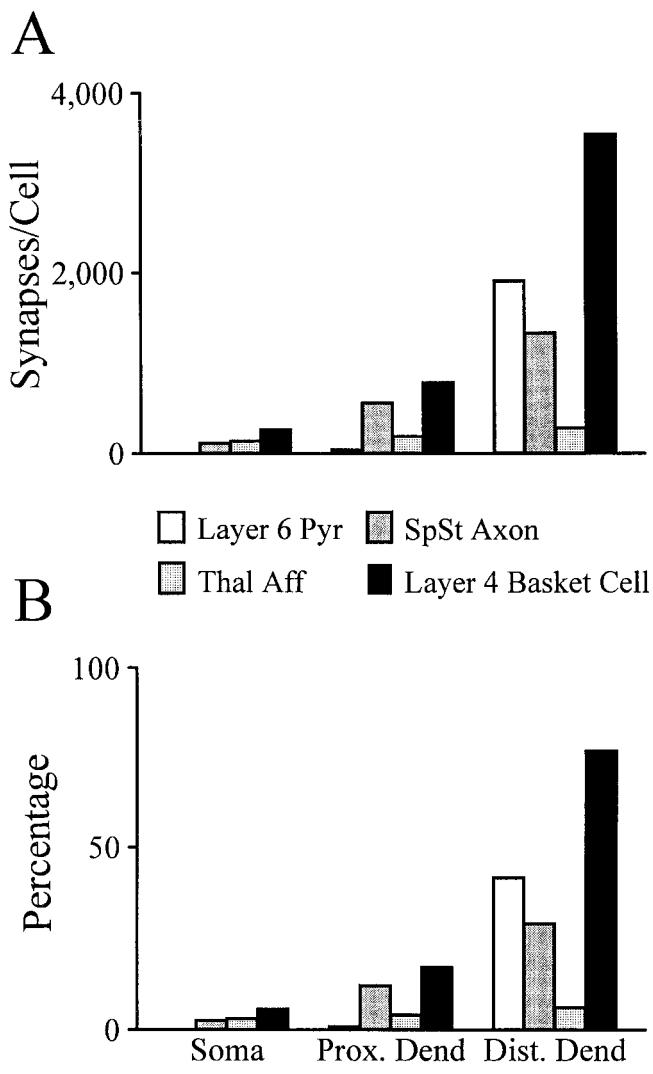

Fig. 7. Histogram of the data showing the relative contribution of the three excitatory sources to the layer 4 basket cell data (top histograms as synapses/cell; lower histograms as a percentage). Abbreviations as in Figure 6.

Only four symmetric synapses were found on the portion of soma sampled. The boutons forming these synapses were much smaller (mean 0.39 μm^2) than the average basket cell bouton (0.66 μm^2). Only six symmetric synapses were found on distal dendrites. These were insufficient to attempt to fit a function. However, sufficient symmetric synapses were sampled on the proximal dendrites to attempt the least-squared fitting procedure. The results are shown in Figure 8. The proximal dendritic data could be fitted statistically to account for only 55% of the variance. This relative imprecision meant that we could only give an upper limit (79%) on the possible contribution of the symmetric synapses from other similar basket cells. The actual figure is likely to be less. From the symmetric synaptic densities, the total number of symmetric synapses formed with basket cells is 338 (soma 50, proximal dendrites 208, and distal dendrites 80), of which at most 267 synapses are from other basket cells.

DISCUSSION

In his thorough review of the basket cells of the visual cortex, Kisvárday (1992) emphasised that the weakest link in the otherwise considerable body of knowledge of the cortical basket cells was "the almost complete lack of knowledge concerning the intracortical input to their dendritic shafts." P395 A partial picture is now emerging, however, for the basket cells of layer 4 of the cat's visual

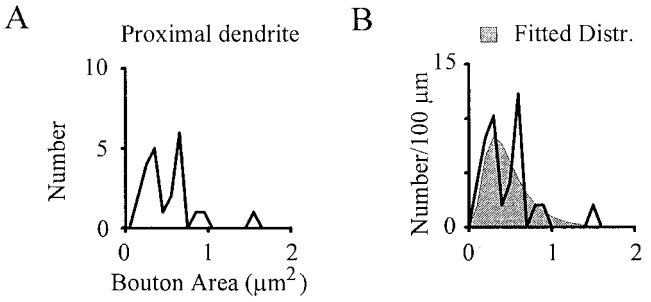

Fig. 8. Distributions and proportions of symmetric synapses formed with the proximal dendrite of basket cells of layer 4 in the visual cortex of the cat. **A:** The cross-sectional area of the boutons making these synapses. **B:** Symmetric synaptic input distributions to the proximal dendrite of basket cells of layer 4. The distributions from Figure 8A are normalised. The filled distributions are from the bouton data of basket cell axons (Fig. 5B). The relative contribution of the symmetric input was determined by a least-square statistical fitting procedure (R = 0.75).

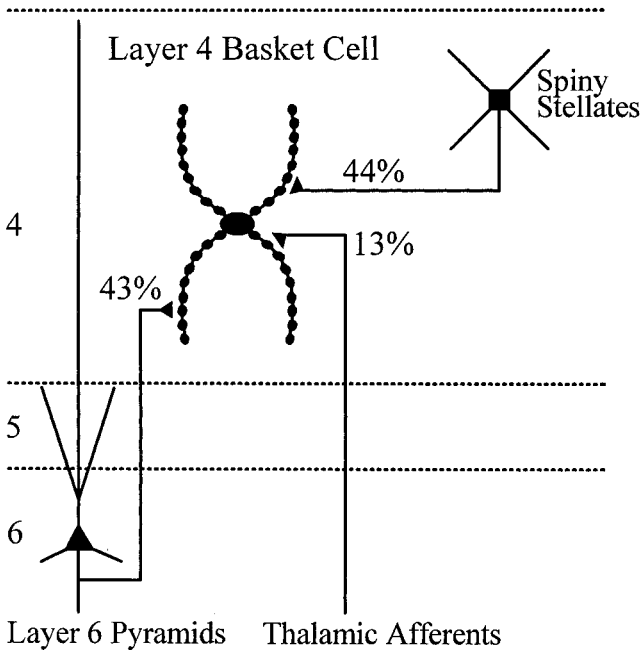

Fig. 9. Schematic of the connectivity to the basket cell of layer 4 with the calculated percentage of asymmetric synapses formed with layer 6 pyramidal cells, thalamic afferents, and spiny stellate cells.

cortex. Our previous work provided electrophysiological evidence that the basket cells of layer 4 of the cat's visual cortex were monosynaptically excited by the thalamic afferents (Martin et al., 1983; Kisvárday et al., 1985; Gabbott et al., 1988). A combined anatomical–physiological study indicated that the thalamic afferents formed asymmetric synapses exclusively with GABAergic somata, and, in addition, formed asymmetric synapses with the dendrites of smooth neurons in layer 4 (Freund et al., 1985). The X and Y axons appeared to innervate different classes of neurons, based on their somal size. McGuire et al. (1984) provided evidence that the putative basket cell dendrites were among the targets of the axonal projection of layer 6 pyramidal cells to layer 4. Their finding was confirmed by Somogyi and co-workers (see Somogyi, 1989). Neurochemically, layer 4 basket cells are GABA-immunoreactive (Freund et al., 1983; Somogyi and Soltész, 1986), as the present study confirms. In addition they may be immunopositive for galactosamine (Naegele and Katz, 1990). At the light microscope level their axons formed very compact arbors, mainly in layer 4, and individual collaterals supplied boutons that made contacts with the somata of other layer 4 neurons, an arrangement that is characteristic of basket cells (Ramón y Cajal, 1911). Our statistical analysis has now provided quantitative estimates of the major excitatory inputs and the main inhibitory input to these basket cells. The analysis has also provided an insight into the selective nature of these inputs: that they form synapses preferentially at certain locations on the basket cells.

In our previous study on spiny stellate cells of layer 4 of the primary visual cortex (Ahmed et al., 1994), we used a "template-matching" procedure to estimate the relative numbers of synapses contributed by the three major excitatory afferents to the main excitatory neuron of this layer. This analysis of the anatomical input to the spiny stellate cells proved to be a strong predictor of the divisions among the physiological classes of synapses recorded in spiny stellate cells of cat area 17 in vitro (Stratford et al., 1996). In the present study we extended the analysis to basket cells, which appear to be the main inhibitory neuron of layer 4. We examined a total of 423 synapses formed with the layer 4 basket cells. By comparing the sizes of the boutons that formed synapses onto the dendrite with the different bouton sizes of the three main excitatory inputs and one inhibitory input to this layer, we were able to estimate the proportion of synapses provided by these different sources (Fig. 9). These percentage values are based on the statistically "best fit" case (minimised χ^2), and allow for the total somal area (506 μm^2), and the total proximal (415 μm) and total distal (2828 μm) dendritic lengths. For the asymmetric data, the three major excitatory sources of the afferents of layer 4 could account for over 90% of the asymmetric synapses formed with the basket cell. However, there was some indication of a contribution of asymmetric synapses from an unidentified fourth source, which contributed to a distinct peak in the distribution at 0.65 μm^2. The peak of this putative fourth source lay at the overlap between spiny stellate and thalamic afferent boutons, i.e., boutons with a cross-sectional area of 0.5–0.7 μm^2. This small peak was not seen in the similar analysis of the spiny stellate cells (Ahmed et al., 1994). One possible source for this peak that should be considered is the claustrum, which projects to layer 4 and forms asymmetric synapses with dendritic shafts and spines. There is no quantitative description of bouton sizes nor measures of density for the claustral input to layer 4 (LeVay, 1986). However, the qualitative description of LeVay (1986) is that the claustral boutons are tiny and are greatly outnumbered by the contribution of the thalamic afferents, which indicates that the claustrum is unlikely to be this putative fourth source. At best the claustrum can contribute only a small percentage of synapses to layer 4. It seems that another type of specific afferent of layer 4 remains to be defined. Given the close correlation between the input determined anatomically and the input determined physiologically for the spiny stellate cells (Ahmed et al., 1994; Stratford et al., 1996), similar physiological analyses of the input to the basket cells may reveal some insight as to the source of the additional excitatory input suggested by the present data.

The intracortical excitatory inputs (from layer 6 pyramids and spiny stellate cells) accounted for 87% of the fitted data for asymmetric synapses, the remainder being from thalamic afferents. Of note is that the layer 6 pyramidal input seems to be strictly restricted to the distal dendrites of layer 4 basket cells. There was no evidence that they formed synapses with the soma. This is consistent with the findings of McGuire et al. (1984), who found that the layer 6 pyramidal cells did not form synapses with the somata and proximal dendrites. The synapses on the distal dendrites were clearly grouped around the dendritic beads. There is some suggestion of this feature in the reconstructions of layer 4 dendrites made by McGuire et al. (1984), although they reported that the synapses did not appear to differentiate between beads and the interbead segments. We do confirm their finding that the beads themselves were created by a cluster of mitochondria within the dendrite, whereas the interbead segments were virtually devoid of mitochondria. It has been debated whether or not the beadedness of the smooth cells is an artefact. On the basis of the present data, the beads are clearly not artefacts, but rather appear to form a functional compartment with dense synaptic input that gives rise to a high metabolic demand. The boutons that formed synapses with the beads were of variable size for a given cluster, so each cluster probably consists of a combination of inputs from different sources. The functional significance of such clustering, which was not seen on spiny stellate dendrites (Ahmed et al., 1994), remains to be determined.

The layer 6 pyramidal cells formed only 5% of all asymmetric synapses with proximal dendrites, and 54% of all asymmetric synapses with distal dendrites. The input to the soma was dominated by the thalamic and spiny stellate synapses (respectively, 55% and 45% of the synapses), and proximal dendrites (respectively, 24% and 71% of all asymmetric synapses). Previous studies of single thalamic axons (Freund et al., 1985; Friedlander and Martin, 1989) had indicated that the somata and the proximal dendrites of smooth or GABAergic neurons were prominent targets of the thalamic afferents. This contrasts strongly with the spiny stellate neurons where the soma and proximal dendrites are virtually devoid of asymmetric synapses (Ahmed et al., 1994).

Most previous studies on the synapses formed with smooth or GABAergic neurons of layer 4 have concentrated on the input to the soma and proximal dendrites (Davis and Sterling, 1979; Hamos et al., 1983; Einstein et al., 1987). Our estimated somal synaptic density of 70 synapses 100 μm^{-2} is almost twice as large as the mean values reported by Hamos et al. (1983), but is similar to the type II class of Davis and Sterling (1979). The ratio of asymmetric to symmetric synapses found here (6:1) is very different from those of these authors, but then a large number of their synapses could not be classified. Admittedly, our estimate is derived from a single soma, but all the studies cited have had to deal with very small samples, because all have used the serial EM technique.

The symmetric input to the basket cell represented only 6% of all synaptic input with a strong preference for the proximal dendrites (61% of all symmetric synapses). In this region there was good matching between the areas of the basket cells boutons and the boutons that formed symmetric synapses with the dendrites of the basket cell (Fig. 8). The boutons that formed the symmetric synapses found on the soma were much smaller than the average boutons of layer 4 basket cells. This dominance of the dendritic over the somatic input for boutons forming symmetric synapses is consistent with our observations that only 20–26% of the synapses formed by the layer 4 basket cells are with somata (Kisvárday et al., 1987; Somogyi and Soltész, 1987; present study). Many of those somata that do form synapses with the basket cell boutons may belong to the spiny neurons of layer 4, whose somata form almost exclusively symmetric synapses (LeVay, 1973; Hornung and Garey, 1981; Davis and Sterling, 1979). In contrast, the somata of these basket cells are a major target of excitatory input from thalamic afferents and spiny stellate cells.

Based on the total somal area, and the proximal and distal dendritic lengths, we calculated that as much as 79% of the symmetric contacts could have been made by axons of other basket cells (Fig. 8). This is likely to be an overestimate, given that we could account for only 55% of the variance. We require bouton size distributions from other inhibitory neuron classes in order to obtain a more realistic estimate, but as yet we have not encountered other classes in layer 4 in our in vivo recordings. More varieties might be encountered with the somewhat easier in vitro technique. In order to give us a more robust estimate, the presynaptic symmetric distribution onto the proximal dendrites of basket cells was examined for secondary peaks. The indication was that there are likely to be two other sources, one with smaller boutons and the other with larger boutons than those from basket cells.

The study most closely related to ours is that of White and Rock (1981), who studied two different types of smooth neurons in layer 4 of the primary somatosensory cortex of the mouse. They found that one type (a bitufted cell) received 20% of its asymmetric synapses from the thalamus, but that only 4% of the synapses were of thalamic origin in another type of "multipolar" neuron. It is possible that these latter neurons are comparable to the basket cells studied here, which also would be classified as multipolar (Peters and Regidor, 1981); however, without a description of the axonal morphology in the mouse we cannot draw firm parallels. Nevertheless, it is clear that in both the mouse and the cat, the proximal dendrites have a high density of synapses, and that in both only a minority of synapses arise from thalamic sources. The evidence presented above establishes that the majority of asymmetric and symmetric synapses arise from other layer 4 neurons and from the layer 6 pyramidal cells. The data from the mouse is also congruent with this conclusion. White and Keller (1987) provided evidence that about 90% of the synapses formed by the recurrent collaterals of corticothalamic pyramidal cells were with smooth neurons in layer 4 of the barrel cortex. Such rich intracortical connections suggest that even in the input stage of the cortex, the local cortical circuits have a strong influence, both through their strong connections with the spiny neurons of layer 4 (Ahmed et al., 1994) and through the basket cells.

The role of the layer 4 basket cells in the function of the neocortical circuits has not been explored directly. However, there are a number of studies that provide indirect evidence of the importance of intracortical inhibition within simple cells. Inhibition has been blocked by $GABA_A$ receptor antagonists (Sillito, 1975, 1977; Tsumoto et al., 1979; Sillito et al., 1980), and has been shown to produce a decrement of subfield antagonism, orientation selectivity, and directional tuning in simple cells. Further suggestive evidence for their role in shaping receptive fields comes from the decrease in end-inhibition that occurs when the

claustrum is ablated (Sherk and LeVay, 1983). The claustrum receives an excitatory projection from layer 6 pyramidal cells that have elongated receptive fields (Grieve and Sillito, 1995). Claustral neurons in turn send a direct projection to layer 4 of area 17, where they form synapses with both smooth and spiny cells (LeVay, 1986). When layer 6 is inactivated by GABA ionophoresis, the length-tuning properties of layer 4 neurons declines (Bolz and Gilbert, 1986). However, the interpretation of these results is complicated by the fact that layer 6 pyramidal cells also project directly to layer 4 and back to the lateral geniculate nucleus, where their action is to increase the surround inhibition of the relay neurons that project to layer 4 (Murphy and Sillito, 1987). Both alternative circuits require the layer 4 inhibitory neurons, however, and the most likely candidates for these in layer 4A are the basket cells we have studied here.

It is extremely rare to find spiny stellate neurons that do not have a simple receptive field (Kelly and van Essen, 1974; Gilbert and Wiesel, 1979; Martin and Whitteridge, 1984; Anderson et al., 1994a,b). Although the sample is relatively small, there are indications that the basket cells of layer 4 vary more than spiny stellates in their receptive field types. We have now recorded several basket cells in layer 4 that have complex receptive fields and one with a non-oriented receptive field (Martin et al., 1983; Kisvárday et al., 1985; present study). Interestingly, both layer 6 pyramidal cells studied by McGuire et al. (1984) were complex cells, and both had strong projections to the distal dendrites of layer 4 smooth cells. This may indicate that simple and complex cells in layer 4 are located in parallel circuits, much as the X- and Y-type thalamic streams in layer 4 remain separate (Bullier and Henry, 1979; Martin and Whitteridge, 1984; Freund et al., 1985). The variability in receptive field type may also indicate a higher degree of convergence of different excitatory sources onto the smooth neurons. Within a particular pathway there is certainly high convergence. For example, even if single thalamic axons form multiple synapses with the basket cells, there must still be a high degree of convergence to account for the total of 616 thalamic synapses formed with the average layer 4 basket cell.

All layer 4 neurons, including the spiny stellate and the pyramidal and smooth neurons, are recipients of direct excitation from the thalamus (Martin and Whitteridge, 1984); but also, as demonstrated here, the basket cells are recipients of disynaptic excitation from other layer 4 neurons and from layer 6 pyramids. In addition, to explain the origin of the functional properties within this layer, the extensive intracortical excitatory input to these neurons from layer 6 pyramids (1947 synapses) and spiny stellates (2022 synapses) needs to be considered (Douglas and Martin, 1991). The symmetric synapses originate mainly from other basket cells and, from the extent of their axonal arborization, these cells probably have spatially overlapping receptive fields. This implies that both the basket cells and their targets will be activated by the same stimulus. This seeming paradox—coactivation of inhibitory and excitatory cells that are mutually connected—is explained by a model of the recurrent circuits of the visual cortex (Douglas et al., 1989, 1995; Douglas and Martin, 1991). Inhibition acts to control the gain of the recurrent amplification inherent in the recurrent excitatory pathways. Because the inhibitory neurons are embedded in a common network, the recurrent inhibition received by a particular neuron is proportional to the activity of the whole network. This proportionality is a characteristic property of cortical neurons; they do not exhibit bistable behaviour, for example. This proportionality can only be achieved by a circuit where inhibitory neurons are recurrently connected with the neurons that excite them, and that they in turn inhibit. Specificity within the network can be achieved by differential activation of the inhibitory neurons by the feed-forward excitatory pathways. This provides for a simple explanation of directionality, for example (Douglas and Martin, 1991; Suarez et al., 1995).

Even in the conventional models of simple cells, inhibitory neurons are required to be connected in a similar manner. For example, one of the most characteristic properties of simple cells is the antagonism between the subfields (Hubel and Wiesel, 1962). In their comprehensive extracellular study, Palmer and Davis (1981) explained this property by a model in which the simple receptive field consisted of co-extensive excitatory and inhibitory regions of opposite signs. They termed this a "push–pull" model because excitation of a subfield was associated with a concomitant reduction in inhibition in the same subfield and vice versa (Palmer and Davis, 1981; Heggelund, 1986; McLean et al., 1994). Our results are consistent with this model since the inhibitory–excitatory interactions that lead to antagonism between the ON and OFF subfields of simple cells can be mediated only by neurons that have virtually overlapping simple receptive fields with the opposite polarity. Thus, on this model it is necessary that the basket neurons are excited by local spiny neurons in layer 4. The physiological properties of the synapses that provide this excitatory input remain unknown, and we are presently extending our studies of the layer 4 basket cells to identifying the physiology of their synaptic input.

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